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FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Transcriptional Regulation of Skeletal Muscle Progenitor Cell Fate

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The Transcriptional Regulation of Skeletal Muscle Progenitor Cell Fate

Josée Savage

A thesis submitted to
The Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry, Microbiology and Immunology
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Ottawa, Ontario, Canada

Thesis Format: Thesis with Self-Standing Chapters

Your file Votre référence
ISBN: 978-0-494-51821-2
Our file Notre référence
ISBN: 978-0-494-51821-2

ABSTRACT

The progression from the undifferentiated state to the fully differentiated skeletal myocyte is orchestrated by a highly complex network of transcription factors whose expression is regulated by several families of molecules. However, not much is known about the early events that lead to the expression of muscle progenitor markers. This series of studies aims to further understand the mechanisms which regulate commitment to the myogenic lineage.

In Chapter 2, Sox15 was found to be sufficient for the expression of pre-skeletal mesodermal markers, but failed to upregulate the expression of the myogenic regulatory factors while Sox7 was able to induce the entire myogenic program. Cells overexpressing Sox15 show elevated levels of genes that inhibit differentiation and promote proliferation, which coincides with the lack of progression past the muscle precursor stage. These results demonstrate that Sox15 and Sox7 have differential roles in regulating skeletal myogenesis.

Chapter 3 demonstrates that the Wnt signaling pathway positively regulates the expression of Foxc1/2. This result was further extended by the demonstration that β-catenin occupies the chromatin upstream of Foxc1 during differentiation. Loss of functional Gli2 and Meox1 led to the downregulation of Foxc1/2, while misexpression of Gli2 upregulated the expression of these two genes. These studies have helped us define the pathways that regulate Foxc1/2 expression, and more specifically that functional β-catenin is required for Foxc1/2 expression.

Chapter 4 investigates the contribution of soluble signaling molecules to the regulation of Sox7 expression. Treatment with retinoic acid accelerated Sox7 and Wnt3a

expression, and activation of the Wnt pathway led to upregulation of Sox7, most likely by direct interaction of β-catenin with the promoter, as demonstrated by chromatin immunoprecipitation. It is likely that retinoic acid acts upstream of the Wnt signaling pathway, since it was unable to overcome the repression of Sox7 expression by a non-functional β-catenin. Therefore, Sox7 can be placed downstream of both the Wnt pathway and retinoic acid signaling during myogenesis in P19 cells.

Theses studies have provided a clearer understanding of the pathways that regulate the expression of genes involved in the regulation muscle precursor cell fate.

ACKNOWLEDGMENTS

I would like to thank Ilona Skerjanc for allowing me the opportunity to pursue my graduate studies in her lab. The advice, support and guidance you have provided me over the years has allowed me to get to where I am today, and for that I am truly grateful.

I also wish to extend my appreciation to my advisory committee members, both at Western (Dr. Ball and Dr. Litchfield) as well as at uOttawa (Dr. Bennett, Dr. Picketts and Dr. Rudnicki). Your advice, suggestions and criticisms contributed positively to my development as a scientist.

To conduct high-quality scientific research, the financial support of granting agencies is indispensable. I have been very fortunate to have received studentships throughout my graduate studies from the Ontario Graduate Scholarship, the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Canadian Institutes of Health Research (CIHR – Institute of Aging).

Time spent working in the lab is always more fun in the presence of great company. To all of the members of the Skerjanc lab which I've had the opportunity to work with, your friendship, technical expertise and moral support are greatly appreciated!

Rien de ceci n'aurait pu se faire sans l'appui et l'encouragement inlassable de mon futur mari Marc, mes parents Jean et Monique et ma sœur Julie. Je ne suis pas capable de dire dans les mots qui conviennent, le rôle que vous avez pu jouer à mes côtés pour en arriver là. Je n'aurais pas pu accomplir ce que j'ai fait sans vous...

Dédié à Marc, Mom, Dad et Julie

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List of Abbreviations

aa	Amino acid
ADH	Alcohol dehydrogenase
ATCC	American tissue type collection
BMP	Bone morphogenetic protein
bHLH	Basic helix-loop-helix
$Ca2^{+}$	Calcium ions
CamK11	$Ca2^{+}$-calmodulin-dependent protein kinase II
CBP	CREB-binding protein
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
CREB	cAMP response element binding
CYP26	Cytochrome P450 26
DEAB	Dethylaminobenzaldehyde
Dhh	Desert Hedgehog
Dll	Delta-like ligand
DM	Dermomyotome
DML	Dorso-medial lip
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E	Embryonic day
EB	Embryoid body

EC	Embryonal carcinoma
EN-2	Engrailed 2
EnR	Engrailed repressor domain
ES	Embryonic stem
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Fz	Frizzled
GFP	Green fluorescent protein
GSK	Glycogen synthase kinase
HDAC	Histone deacetylase
Hh	Hedgehog
HMG	High mobility group
Ihh	Indian hedgehog
JNK	Janus kinase
KO	Knockout
LDL	Low-density lipoprotein
Lnfg	Lunatic fringe
MCK	Muscle creatine kinase
MHC	Myosin heavy chain
MRF	Myogenic regulatory factor
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
PCP	Planar cell polarity

PGK	Phosphoglycerate kinase
PI	Phosphotidylinositol
PKA	Protein kinase A
PKC	Protein kinase C
PSM	Pre-somitic mesoderm
Ptch1	Patched 1
Puro	Puromycin
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP	Retinol binding protein
RDH	Retinol dehydrogenase
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
Shh	Sonic hedgehog
Smo	Smoothened
Sp	Splotch
SSC	Sodium citrate/sodium chloride buffer
T	Brachyury-T
TA	Tibialis anterior

LIST OF FIGURES

Contribution of Collaborators

Some of the materials contained in this thesis are from submitted manuscripts. The authors that contributed to this work are as follows.

In Chapter 2:

- Andrew Conley, an honours student, originally created the P19[Sox15] cell lines (although none of his clones were used for any of the experiments shown here).

- Sophie Boisvenue provided technical assistance in the making of the P19[Sox15/EnR] cDNA as well as the generation of the P19[Sox/EnR] cell lines.

- Dr. Alex Blais provided protocols and helpful suggestions for the ChIP experiments (although all experiments were performed by myself), and assisted with the microarray analysis

In Chapter 3:

- the P19[b-catenin/EnR] and P19[Gli/EnR] clonal cell lines were generated by Helen Petropoulos.

- Alan Ridgeway created the P19[Meox/EnR] cell lines.

- Xionan Wang created the P19[Gli2] cell lines, and Anastassia Voronova performed the differentiations in Figure 5, while the Q-PCR analysis was performed by myself.

- All other experiments were performed by myself.

In Chapter 4:

- Karen Kennedy prepared the RNA samples for microarray, while I performed the data analysis which identified Sox7 as an RA-responsive gene.

- The P19[b-catenin/EnR] cell lines were generated by Helen Petropoulos.

- All of the experiments presented in this chapter were performed and analyzed by myself.

The manuscripts for Chapters two, three and four were written by me, with assistance from Ilona Skerjanc.

Chapter 1

Introduction

1.1 – Vertebrate Embryonic Skeletal Muscle Development

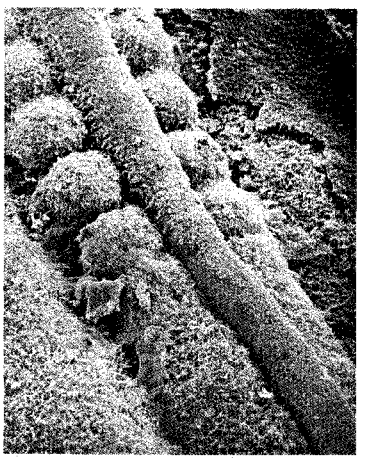
In order for proper embryonic growth and development to occur, cells need to be able to make decisions regarding the choice of lineage commitment, proliferation, differentiation and death. These decisions are regulated by a complex spatial and temporal network of transcription factors and signalling molecules which regulate all embryonic patterning processes.

The process of embryonic skeletal muscle formation begins during gastrulation, which marks the transition from a simple, not very highly organized group of cells to a more complex, multilayered embryo with three distinguishable germ layers, the endoderm, mesoderm and ectoderm (115). At this stage, highly coordinated movements control the invagination of cells through the primitive streak. Cells which migrate medially along the anterior-posterior axis are patterned to become paraxial mesoderm, while cells that travel further along the medial axis become lateral plate mesoderm, a source for blood, cardiac and vascular derivatives (190)

As the primitive streak regresses, the paraxial mesoderm is further patterned into somites, which are blocks of segmented mesoderm that form symmetrically on each side of the neural tube (Figure 1.1, A). Somitogenesis proceeds in a rostral-caudal fashion and is regulated by a "clock-wavefront" model, originally proposed more than thirty years ago, in which a clock sets the pace for somite formation and a wave of maturation of the pre-somitic mesoderm (PSM) (48). It was later discovered that one function of the Notch and Wnt signalling pathways is to control the cyclic expression of genes during somitogenesis, oscillations of which renders cells competent to respond to the wave of determination that establishes boundaries between somites. The wave of determination consists of a gradient of

Figure 1.1 – Vertebrate somitogenesis. Panel A: Scanning electron micrograph depicting the anterior to posterior segmentation of the paraxial mesoderm into symmetrical pairs of somites flanking the neural tube. Copyright: Gilbert S.F., *Developmental Biology 6th ed*, 2000, Figure 14.3. Panel B: Schematic depiction of the somite segmentation process in vertebrates. The paraxial (pre-somitic) mesoderm is segmented in an anterior (rostral) to posterior (caudal) fashion, forming ball-like structures along the neural tube/notochord. Secreted factors from the surrounding tissues provide signals for the further patterning of the somite into the ventral sclerotome and the dorsal dermomyotome. Cells migrating first from the dorsomedial lip, followed by migration from all four borders of the dermomyotome, populate the myotome, located under the dermomyotome, forming a continuous sheet of epaxial and hypaxial muscle. Adapted from reference (30) with permission from Wiley InterScience © 2003.

A



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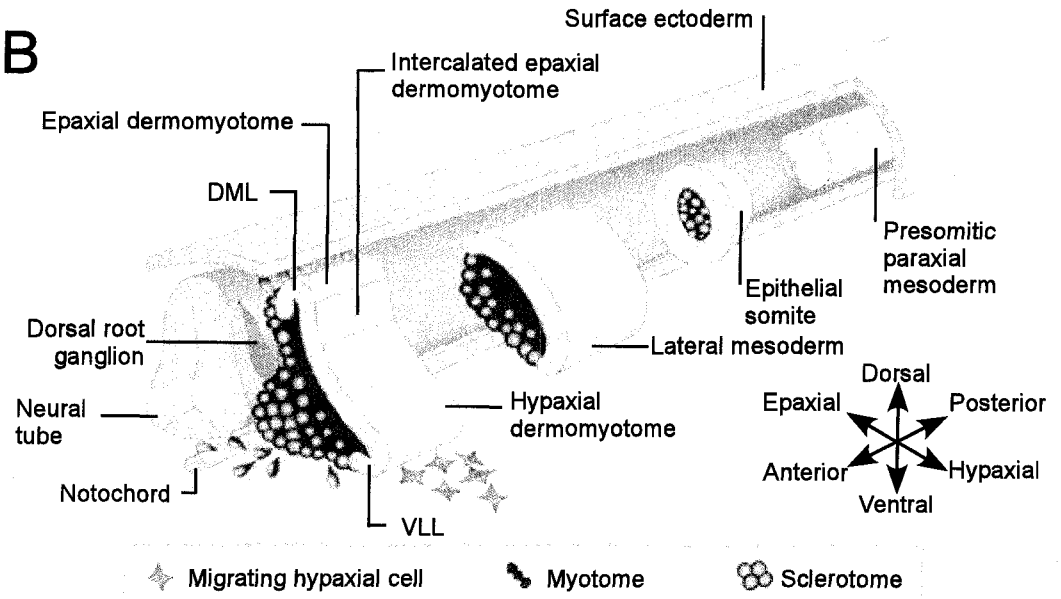


fibroblast growth factor 8 (Fgf8) signalling, which decreases in a caudal-rostral manner, and an antagonistically expressed gradient of retinoic acid. When Fgf8 levels decrease below a certain threshold, PSM cells are now competent to respond to the signals from the clock, thus forming segmental boundaries (reviewed in 53).

Following segmentation, the differentiation of epithelial somites into the dermomyotome (DM) and the myotome compartments are influenced by signals such as Wnt and Shh emanating from the surrounding embryonic tissues, including the notochord, neural tube and overlying ectoderm (reviewed in (30) (Figure 1.1, B). The DM is the dorsal compartment of the somite which gives rise to muscles of the trunk, while the sclerotome is the ventral portion of the somite which eventually separates to give rise to the bones of the axial skeleton. Wnt and Sonic hedgehog (Shh) signalling results in the DM-specific expression of transcription factors such as Pax3, Pax7, Meox1 and Gli2, which characterize the muscle precursor population. These transcription factors in turn regulate the expression of the myogenic regulatory factors (MRFs), genes which control differentiation and confer onto the cell its myogenic identity (reviewed in 30). The molecular events involved in muscle development are summarized in Figure 1.2. Cells from the DM migrate to form the myotome, the site of the first fully differentiated embryonic myocyte. It is now accepted that the myotome is generated in two steps, primary and secondary myotome formation, the first of which involves migration of cells from the DM to the underlying layer. Using electroporation of a GFP-reporter, Gros et al have demonstrated that the first phase of primary myotome formation involves the migration of cells through the dorsomedial lip (DML), followed by elongation of the cells across the rostral caudal borders of the somite. As new myoblasts come through the DML, they displace the older myocytes. During the second phase of primary myotome formation, cells from all four borders of the DM migrate

Figure 1.2 – The molecular regulation of skeletal muscle development. This drawing is a schematic representation of the series of events involved in patterning the mesoderm into skeletal muscle. The important transcription factors and muscle structural genes have been identified on this diagram.

to the myotome and elongate along the anterior-posterior axis of the compartment (73). Formation of the secondary myotome, which gives rise to the bulk of the body-wall muscles, involves the proliferation and differentiation of muscle progenitors, which migrate directly from the DM into the myotome (73, 169).

Numerous studies have been performed to date with the aim of identifying families of transcription factors which regulate the process of muscle differentiation. Although much is known about the transcriptional networks regulating myogenesis, the mechanisms which regulate the expression of these genes still remain unclear. The following sections provide an overview of our current knowledge of myogenesis, and highlight the questions which still need to be addressed.

1.2 – Transcriptional Regulation of Skeletal Myogenesis

1.2.1 – The Myogenic Regulatory Factors

The myogenic regulatory factors (MRFs) make up a family of conserved basic helix-loop-helix (bHLH) transcription factors which are central to the acquisition of myogenic identity and differentiation into skeletal muscle tissue (217). These include MyoD (Myf3) (51), Myf5 (24), Myogenin (Myf1) (55) and MRF4 (Myf6/Herculin) (23, 134).

The MRFs are expressed in a sequential manner during embryonic development. Myf5, the first to be expressed, localizes to the DML of the epaxial domain in E8.0 embryos, followed by expression of Myogenin at E8.5 (154, 186). MRF4 expression is detected in the myotome from E9.0 to E12.0, after which it is repressed until after birth (84). MyoD is expressed at E10.5, most prominently in the hypaxial domain of the DM (96, 186).

The importance of the MRFs in imparting myogenic potential to cells became evident following experiments in which the MRFs were introduced into non-muscle cell types. Forced expression of MyoD leads to the conversion of other cell types into muscle, most likely by chromatin remodeling at muscle-specific loci (46, 51). MyoD interacts with Brg1, the ATPase subunit of the SWI/SNF chromatin remodeling enzyme (52), as well as PCAF and p300 which acetylate histone DNA, as well as MyoD and are needed for myogenic conversion of non-muscle cell types (162, 185). In addition, MyoD can interact with HDAC1, preventing premature differentiation of myoblasts (120).

Inactivation of MRF expression in mice has revealed a complex hierarchical relationship between the four transcription factors MyoD, Myf5, Myogenin and MRF4. Mice null for MyoD do not display any gross muscle defect, but do upregulate the expression of Myf5 and display delayed hypaxial myogenesis, whereas epaxial myogenesis is unaffected (96, 178). Targeted mutation of the Myf5 allele in mice does not affect myogenesis, however, rib abnormalities are observed (25). Closer examination of $Myf5^{-/-}$ mice revealed a delay in epaxial myogenesis, while limb muscle development was normal (96). Genetic ablation of both MyoD and Myf5 results in the absence of skeletal myoblasts and muscles (180). Taken together, these observations suggest that while MyoD and Myf5 may have redundant functions during myogenesis, they also each have distinct roles in regulating hypaxial and epaxial muscle formation. Insertion of Myogenin into the Myf5 locus in $Myf5^{-/-}/MyoD^{-/-}$ mice rescued the rib defects, but did not restore myogenesis, suggesting that Myf5 action is required early during differentiation (213, 214). In addition, expression of MRF4 in MyoD/Myf5 double mutant mice was sufficient to rescue myoblast determination, suggesting that MRF4 is involved in the early steps of myogenesis (99). Further characterization of MRF mutant animals revealed that $MRF4^{-/-}$ mutant embryos

undergo apparently normal skeletal myogenesis (157), while Myogenin$^{-/-}$ mutants display inhibition of muscle differentiation, despite the continued expression of MyoD (79, 146). Therefore, Myogenin appears to function downstream of Myf5 and MyoD to activate muscle gene expression. Also, MRF4, but not Myf5, was able to rescue skeletal myogenesis in the absence of Myogenin, implying that in addition to its involvement in myoblast determination, MRF4 is also able to regulate differentiation (232). This analysis of MRF mutants has revealed the existence of two classes of MRF: the primary MRFs, Myf5 and MyoD, which are involved in myogenic determination, and the secondary MRFs, MRF4 and Myogenin, which mainly regulate differentiation.

1.2.2 – Pax3 and Pax7

Pax3 belongs to the *Pax* gene family which encodes a group of nine proteins in mammals (106). The Pax3 protein is comprised of an octapeptide motif, a homeodomain and a paired domain which is essential for sequence-specific DNA binding (41, 70). The Pax3 protein sequence is highly conserved among vertebrates, with the chick and mouse sharing 97% similarity at the amino acid level (69). In addition to a highly conserved protein sequence, the mouse and chick homologs of Pax3 also display a remarkable degree of similarity in expression patterns and protein function. In mice, Pax3 transcripts are initially transcribed in the PSM and are expressed throughout the entire epithelial somite prior to being restricted to the lateral domain of the DM (15, 70). Similarly to mouse embryogenesis, Pax3 is detected in the chick somite, and as the epithelial somites detach from the segmental plate, Pax3 expression becomes restricted to the dorsal area of the somite, which will become the future DM (68, 221). Pax7, which is closely related to Pax3, is also expressed in the DM, however its expression is higher in the medial compartment of the DM compared to Pax3

which is predominantly expressed in the lateral portion (68, 95). The role of Pax3 during skeletal muscle development in the embryo has been extensively characterized by examining embryos in which Pax3 function or expression has been altered. Several studies performed using *Splotch* (Sp) mutant mice, in which mutations result in the expression of a non-functional Pax3 protein, have identified a pivotal role for Pax3 in regulating the migration of limb muscle precursor cells. In these animals, limb muscles fail to differentiate due to the lack of migration of the presumptive limb muscle precursor cells from the hypaxial myotome (15, 50, 67). Replacement of one Pax3 allele with a dominant-negative form of Pax3 (Pax3/EnR), results in a milder phenotype than that generated by the complete loss of Pax3 function. In these animals, there is partial preservation of the hypaxial somite, but the expression of Myf5 is compromised. These experiments led to the identification of a binding site within the Myf5 enhancer which is occupied by Pax3 and is needed for proper Myf5 expression in the developing hypaxial somite (4). In addition to regulating the formation of limb muscle precursors, Pax3 is involved in the generation of the deep-back muscles which are derived from the medial-half of the somite. In mice null for both Myf5 and Pax3, epaxial myogenesis is completely ablated and there is no detectable expression of MyoD in the trunk or limbs, indicating that the muscle precursor population is absent in these mutants. Similarly, MyoD is absent in the hypaxial somites and severely impaired in the epaxial somite of *Sp* mice (19). These results indicate that Pax3 is required for the activation of MyoD (200). Pax7 mutants show no overt embryonic skeletal muscle defect, while mutations in Pax3 result in the loss of the hypaxial DM structure. Loss of both Pax3 and Pax7 results in a more severe phenotype than the one observed for the single mutants. In these double-null animals, the skeletal muscles of the trunk are severely compromised and the muscle progenitors undergo continued apoptosis as development proceeds (169).

Recently, several groups have identified a novel population of $Pax3^+/Pax7^+$ cells derived from the central domain of the DM, which continue to proliferate and persist throughout embryonic development (9, 73, 100, 169). These $Pax3^+/Pax7^+$ cells are a source of resident muscle progenitor cells for embryonic and fetal myogenesis, as well as satellite cells which regulate post-natal and adult myogenesis (73, 169). The presence of Pax3 and Pax7 in this cell population is important for conferring the myogenic potential of the muscle progenitors: in the absence of both Pax genes, these cells do not undergo myogenesis (169). Studies have identified a role for both Pax3 and Pax7 in the specification of satellite cells (168, 188). Mice harboring dominant-negative forms of Pax3 (Pax3/EnR) and Pax7 (Pax7/EnR) exhibit downregulation of MyoD, but not Myf5, suggesting that in adult satellite cells, Pax3/7 regulate myogenesis via a MyoD-dependent pathway (168). Together, the studies reviewed here indicate that Pax3/7 contributes to embryonic and fetal myogenesis as well as to muscle regeneration in the adult.

1.2.3 – Meox1

Meox1 and Meox2 (formerly Mox-1 and Mox-2) belong to the large family of homeodomain transcription factors which regulate a variety of developmental processes. Both Meox1 and Meox2 were first isolated in mouse by screening an E8.5 cDNA library using a fragment of endoderm-specific frog homeobox gene (34). Since then, Meox1 and Meox2 homologs have been cloned and characterized in human, chick and zebrafish (36, 60, 66, 71, 148, 166). Meox1 and Meox2 most likely originated from a single ancestral gene: in fact, the cloning and characterization of a prototypical Meox gene in the cephalochordate Amphioxus (the closest living relative to vertebrates), revealed 85-90% similarity in the homeobox with vertebrate Meox genes (135). In addition to sharing highly conserved

sequence homology, Meox1 and Meox2 expression patterns and gene function are also highly conserved in all organisms studied to date. In mice, Meox1 is first detected in the presumptive paraxial mesoderm on embryonic day 7.0, which coincides with gastrulation. As development proceeds, Meox1 transcripts are expressed throughout the entire epithelial somite and, eventually, its expression is restricted to the dermomyotome and the sclerotome. By E11.5, Meox1 is no longer detected in the myotome compartment, suggesting that downregulation of the gene must occur for differentiation to proceed (34, 35). The onset of Meox2 expression, however, is detected at a later stage than that of Meox1. Meox2 transcripts are not observed in the pre-somitic mesoderm, but rather, are first detected at the same time as the onset of somite epithelialization (167). In contrast to Meox1 whose expression decreases as somites mature, Meox2 is maintained at high levels in differentiating somites (167). In addition, Meox2, but not Meox1, is detected at the level of the limb buds, suggesting a role for Meox2 in the development of limb muscle (122). A significant understanding of Meox gene function has been elucidated from the study of single and double homozygous null mice. Meox1 null mice display no apparent defects in skeletal myogenesis, although they possess significant abnormalities of the axial skeleton including rib, vertebral and craniovertebral fusions (122). An important role for Meox2 in the regulation of limb muscle formation has been discovered by studying the phenotype of $Meox2^{-/-}$ mice. These mice display an overall reduction in limb muscle mass, yet no major defects in body wall musculature was observed. Furthermore, the loss of Meox2 led to a decrease in Pax3 expression in myoblasts delaminating from the DM, and a selective reduction of Myf5 positive cells in the limbs, but not in the trunk (122). Embryos null for both Meox1 and Meox2 alleles display a severe depletion of hypaxial and epaxial muscles,

suggesting that the combined function of both Meox gene products is essential for normal formation of the dermomyotome (123).

1.2.4 – Foxc1

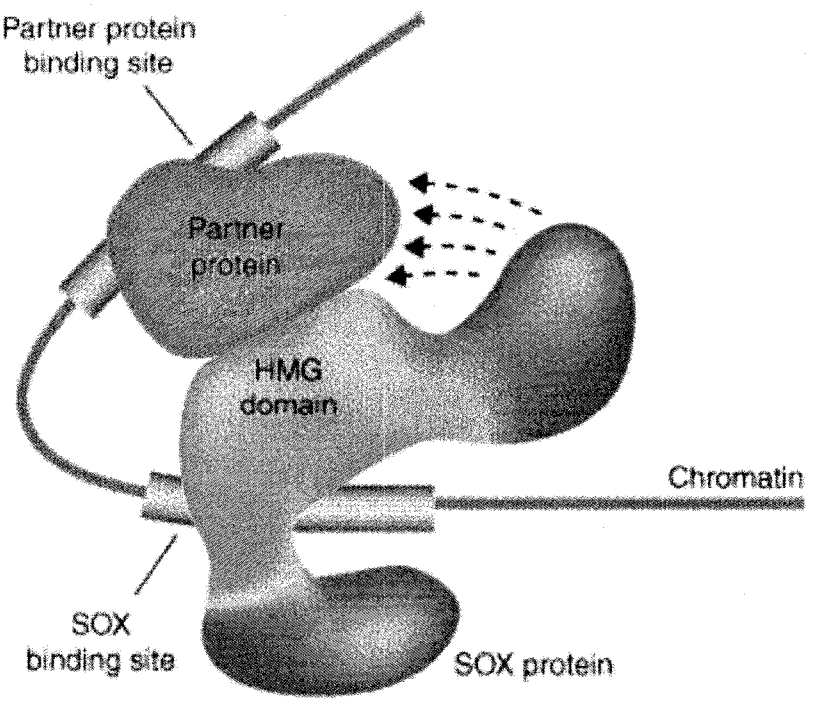
The high complexity of transcriptional regulation of gene expression is achieved in part by the large diversity in DNA binding motifs. One such motif, the one hundred amino acid "winged-helix" domain mediates the DNA binding properties of over one hundred proteins in species ranging from mouse to man (26, 102). Foxc1 and Foxc2 are two closely related members of the *forkhead* family of winged-helix proteins. Fox family members bind DNA as monomers inducing a dramatic bend (80-90 degrees) in the DNA (161). Target DNA sequences are usually comprised of a seven nucleotide core RYMAAYA (R=A or G, Y=C or T, M=A or C). However, this core sequence is not sufficient for high affinity binding, but also depends on flanking sequences on both sides of the core (103). Some groups of *forkhead* proteins have nearly identical DNA binding domains. They are so similar in fact that the specificities might be assumed to be the same. One example is Foxc1 and Foxc2 which share 97% amino acid homology in the forkhead domain (109). Foxc1 and Foxc2 are dynamically expressed in an overlapping pattern in the mesenchymal and endothelial cells of the heart and branchial arches as well as in the paraxial mesoderm and somites (83, 97, 136, 225). Mice lacking either Foxc1 or Foxc2 die pre- and peri-natally and display mild ocular, skeletal and cardiovascular defects (87, 90, 109, 225), whereas $Foxc1^{-/-}$ /$Foxc2^{-/-}$ double mutants display a more complex array of developmental defects. These include cardiovascular defects such as a smaller heart and a drastic disorganization of blood vessels, a complete lack of epithelial somites and segmented paraxial mesoderm. Further analysis of these double null mutant mice revealed that the defect is due to the medial

expansion of the intermediate mesoderm at the expense of paraxial mesoderm, suggesting that a loss of *Foxc* function leads to the increased lateralization of the tissue observed. In support of this hypothesis, ectopic expression of either Foxc1 or Foxc2, in a region of the chick primitive streak fated to form intermediate mesoderm, is sufficient to induce the expression of the somite markers Paraxis and Pax7 (222). Therefore, it appears that Foxc1 and Foxc2 are not only necessary, but also sufficient, to promote paraxial mesoderm cell fate at the expense of other tissues.

1.2.5 – Sox transcription factors

Sox genes are a subgroup of the HMG-box superfamily (111). Proteins are designated to the SOX family if their 79 aa HMG domain contains more than 50% similarity to the HMG domain of Sry, the testis determining factor (111, 147). Sry, the gene responsible for male differentiation on the Y-chromosome, was the first Sox family member identified (74, 192). The 20 known Sox genes in mice/human can be further subdivided into 8 groups (A-H) and two subgroups (B1 and B2) which display 70-95% homology in the HMG domain within groups (187). The evolutionarily conserved HMG motif mediates binding to specific DNA sequences [(A/T)(A/T)CAA(A/T)G] of the minor groove and induces dramatic bending, exposing the major groove of the double helix (47, 58, 78, 118). Given its ability to induce large conformational changes in the target DNA structure, it has been proposed that Sox genes might act as architectural proteins by organizing the local chromatin structure and assembling transcription factors into large, multi-protein complexes (218, 226) (Figure 1.3). Sox genes are ubiquitously expressed in most cell types and tissues

Figure 1.3 – A model for the action of Sox transcription factors. Sox transcription factors may function by interacting with partner proteins which bind adjacent sites in the promoter/enhancer regions of target genes. This interaction could serve to stabilize binding of the transcription factor complex to the DNA. The tissue-specific expression of the Sox interacting protein may contribute to the specificity of gene regulation by the same Sox in different cell types. Reprinted from reference (223) with permission from Elsevier © 2002.



and are involved in many developmental processes including sex determination, neural development, lens development, chondrogenesis and haematopoiesis (215). It appears that specificity is dependent on the interaction of Sox transcription factors with tissue-specific co-factors (98).

Sox15, and its human homolog SOX20, belong to the G-subgroup of HMG-box transcription factors (187, 210). Mouse Sox15 was initially identified in a screen for genes which were differentially expressed in C2C12 myoblasts and myocytes. Sox15 is ubiquitously expressed in adult tissue, but is most abundant in brain and skeletal muscle (10, 113). Sox15 transcripts can also be detected by RT-PCR in whole E8.5 embryos, as well as in ES cells and mouse primary myoblasts (10, 113, 126). Overexpression of Sox15 in the C2C12 myoblast cell line inhibits myogenic differentiation, as demonstrated by the absence of MyoD and Myogenin-positive cells. Sox15 is also able to antagonize activation of the muscle creatine kinase (MCK) promoter in C2C12 cells, while ectopic expression in Swiss 3T3 cells is not sufficient to induce expression of MyoD, Myf5 or Myosin Heavy Chain. This suggests that Sox15 may act as a negative regulator of myoblast differentiation (10, 113). Mice null for Sox15 are viable and show no gross anatomical defects or differences in skeletal muscle mass. However, these mice do display attenuated muscle regeneration following crush injury to the tibialis anterior as well as an 80% reduction in the number of satellite cells, compared to wild type litter mates (113, 131). Consistent with a role in regulating muscle precursor and satellite cell fate, Sox15 has been shown to bind the Foxk1 gene in C2C12 cells and regulate its expression. A role for Foxk1 has been demonstrated in regulating the cell cycle of muscle precursor cells (62, 80). Furthermore, targeted knockdown of Sox15 using RNAi in a myoblast cell line reveal that Sox15 is crucial for maintaining cellular proliferation and promoting proper cell cycle kinetics (131). Taken

together, these studies highlight an important role for Sox15 in regulating muscle precursor cell fate, although the exact mechanisms by which this is achieved are not yet fully understood.

Sox7 belongs to the F-group of the Sox family, along with Sox17 and Sox18 (187). Sox7 homologs have been isolated and identified in mice, humans, xenopus and zebrafish (101, 201, 203). Studies performed using the F9 EC cell model have implicated Sox7 in the regulation of parietal endoderm differentiation. In this cell system, Sox7 regulates endodermal cell fate via the induction of GATA4, GATA6, and Fgf3 activation (59, 145). Recent studies using human embryonic stem cells (hES) have shown that stable expression of Sox7 is sufficient to induce the formation of endoderm progenitor cells (189). Sox7 has also been implicated in regulating cardiogenesis and vascular development by controlling arteriovenous specification in Xenopus and zebrafish (40, 82, 158). The onset of Sox7 transcription can be detected in the somite of mice as early as E7.5-8.0, suggestive of a role in cell fate specification (201). Studies in Xenopus have shown that xSox7, as well as inducing endodermal derivatives, can also induce the expression of mesoderm by regulating expression of the Xnr1-6 and Mixer genes (230). Therefore, we hypothesize that Sox7 may be involved in regulating mesodermal cell fate during embryogenesis, and more specifically, during myogenesis.

1.3 – Signalling Molecules That Regulate Myogenesis

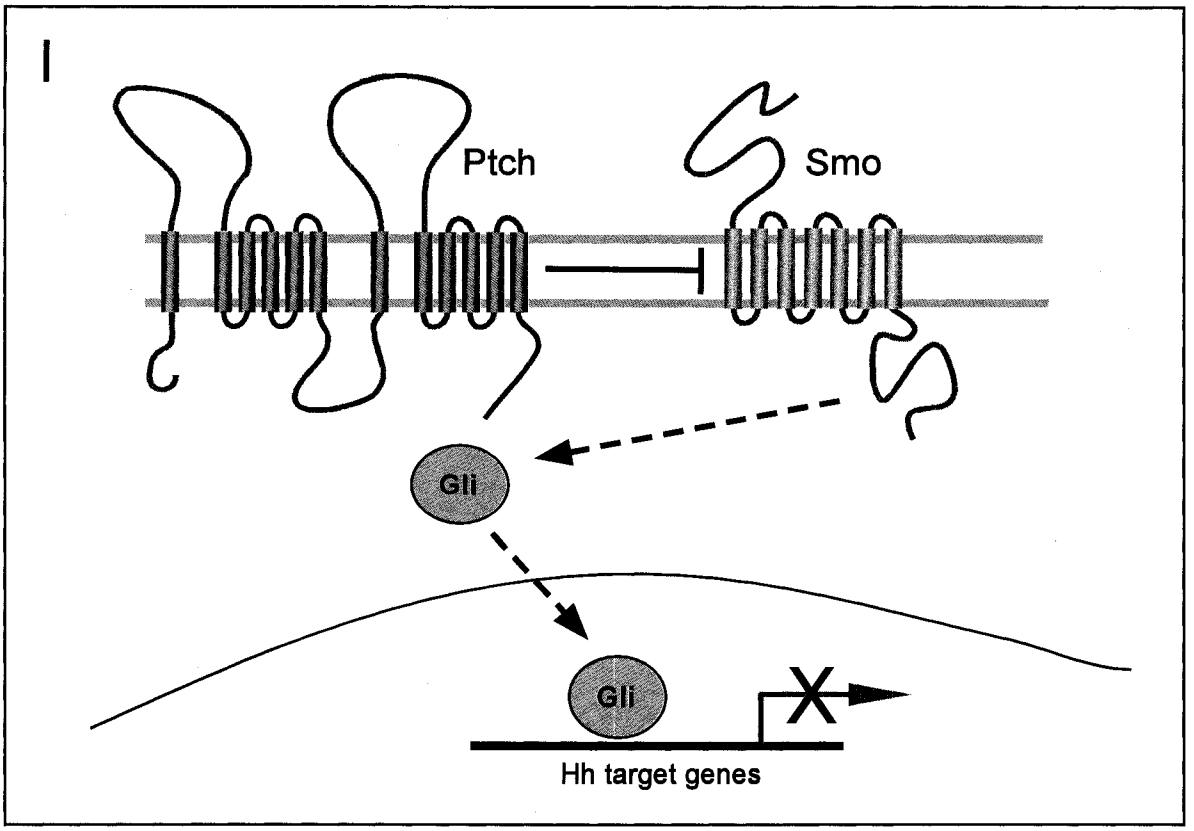
1.3.1 – Shh

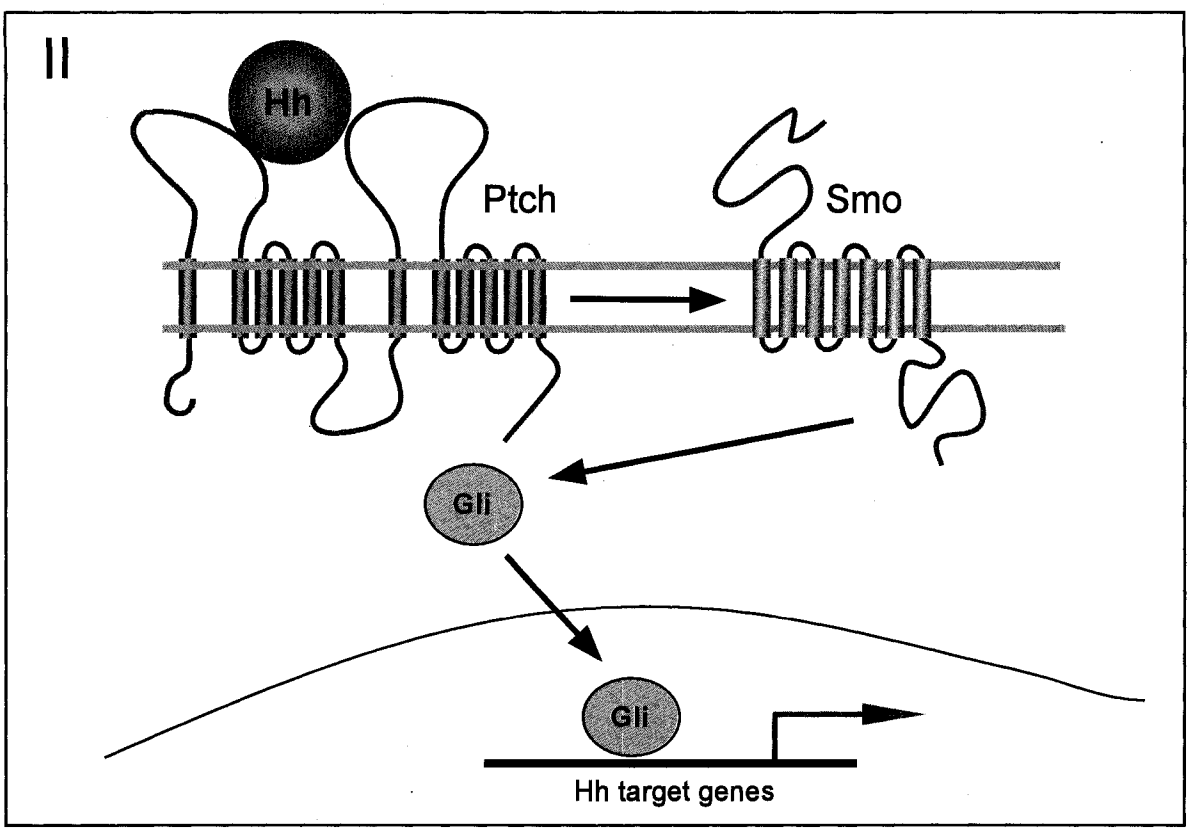
In mammals, the *hedgehog* family of signalling molecules is composed of three members: Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh).

Hedgehogs signal by binding to the cell-surface receptor Patched-1 (Ptch1) (124). In the absence of ligand, Ptch1 inhibits the transmembrane protein Smoothened (Smo), which results in the retention of Gli2 and Gli3 in the cytoplasm. It is believed that Gli2 is degraded under certain cellular contexts, but also functions as a repressor of Hh-target genes (13, 130). Gli3 on the other hand is partially cleaved by removal of its activation domain, which allows it to accumulate in the nucleus where it functions as a transcriptional repressor (212). Binding of Shh to its receptor relieves Smo inhibition, leading to the activation of the Gli transcription factors, which include the activators Gli1/2 and the repressor Gli3. Activated Gli proteins then accumulate in the nucleus where they can regulate gene expression (reviewed in 92) (Figure 1.4). Shh is expressed in several tissues during embryonic development, most notably in the notochord, floor plate of the neural tube and the limb bud (94, 125).

During development, signals from the axial structures (notochord and neural tube) are required to induce myogenesis in the somites. In the absence of axial structures, Shh, in combination with Wnt1 or Wnt3 (see section 1.3.2), is sufficient to enhance myogenesis in somitic tissue explants (144). Similarly, implantation of Shh beads or cells is sufficient to induce MyoD expression in avian somites and allow muscles to develop, most likely by regulating the survival of MyoD-expressing cells (21, 204). Myf5 has been identified as a direct target of long-range Shh signalling through positive regulation by Gli transcription factors (75). Shh, which is required for both Myf5 and MyoD expression, does not induce MyoD expression in the absence of Myf5, suggesting that Myf5 is directly regulated by Gli proteins (20). In support of this hypothesis, proper Myf5 expression is dependent on a Gli binding site within the Myf5 epaxial enhancer (75). Further characterization of the Shh signalling pathway reveals that Gli2 or Gli3 are required for Myf5 activation in epaxial

Figure 1.4 – Mechanism of Shh signaling. In the absence of Shh ligand, the Ptch receptor inhibits Smo, which leads to the degradation and/or cleavage of Gli transcription factors. Under these circumstances, some Gli family members can accumulate in the nucleus and negatively regulate the transcription of Shh-target genes (Panel I). Upon binding of Shh to its receptor Ptch, Smo inhibition is relieved, and Gli transcription factors accumulate in the nucleus to direct the activation of Shh-responsive genes (Panel II).





muscle progenitors, whereas Gli3 represses Myf5 expression in the absence of Shh ligand. Furthermore, $Gli2^{-/-}/Gli3^{-/-}$ double mutant mice display mispatterning of the hypaxial and myotomal genes (130). Taken together, these observations suggest that Gli2 and Gli3 have both positive and negative roles in regulating Myf5 activation. Therefore, Shh secreted from the axial midline is an essential morphogen responsible for the acquisition of myogenic cell fate.

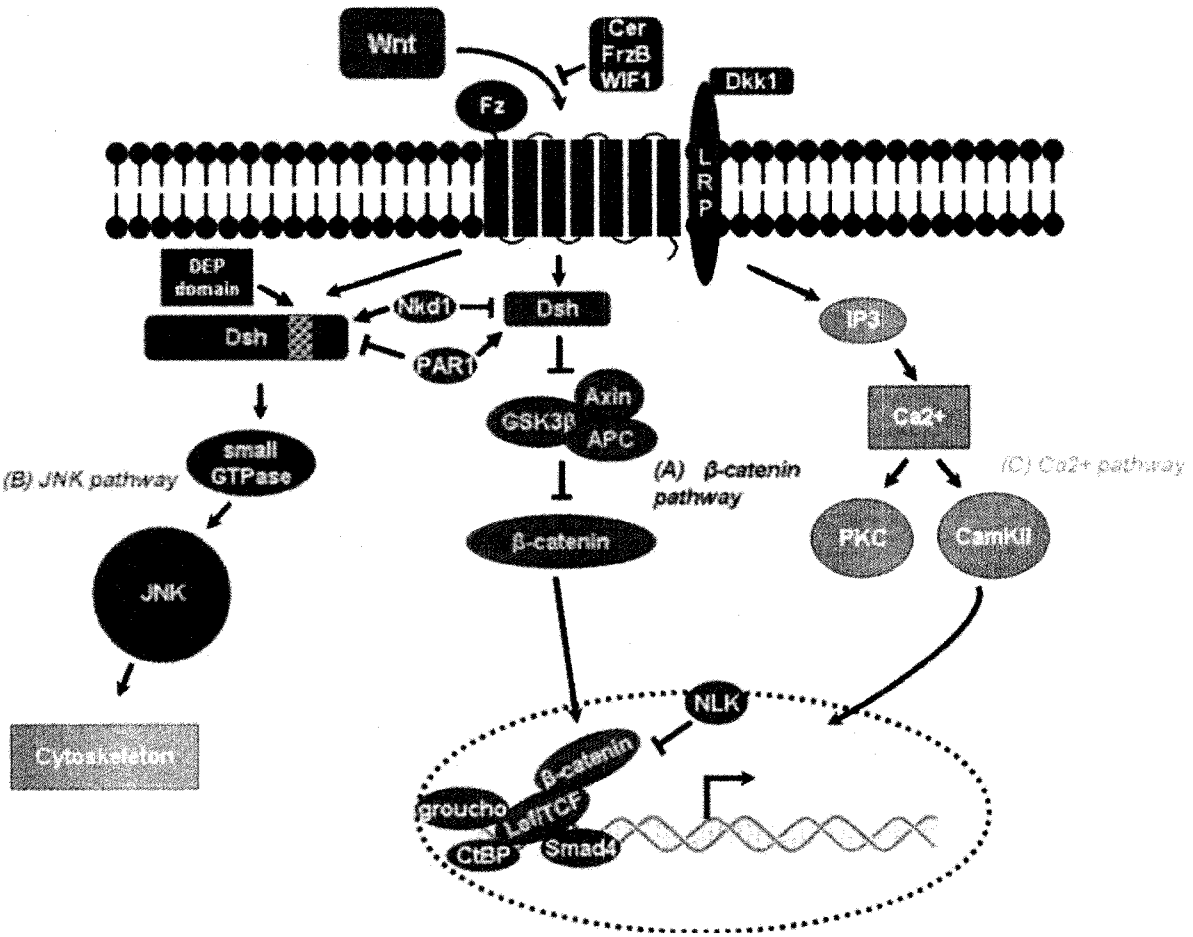
1.3.2 – Wnt

The name Wnt is derived from a combination of wingless (Wg), a Drosophila segment polarity gene, and Int-1 which is the mouse homolog of wingless (5, 173). The Wnt family, composed of nineteen mammalian Wnt homologs, is able to transduce its signals via three separate mechanisms: the canonical signalling pathway, the planar-cell polarity pathway (PCP) and the Wnt/Ca2+ pathway (Figure 1.5).

1.3.2.1 – Mechanisms of Wnt signalling

During canonical signalling, Wnt functions by binding to seven-pass transmembrane cell surface receptors of the Frizzled (Fz) family (12). Recent studies have also implied that Fz-mediated transduction of the canonical Wnt signal requires a presence of a co-receptor, LRP5 or LRP6, which are single-pass transmembrane proteins of the low-density lipoprotein (LDL) family (116, 216). In the absence of Wnt ligand, cytoplasmic β-catenin associates with APC and GSK3β to form the destruction complex. In this complex, GSK3β phosphorylates β-catenin, which then becomes ubiquitinated and targeted for degradation via

Figure 1.5 – Mechanisms of Wnt signal transduction. The Wnt signal can be propagated via three distinct mechanisms. (A) Activation of the canonical Wnt pathway leads to the accumulation of β-catenin in the nucleus, which positively regulates transcription of Wnt-target genes. (B) The planar cell polarity pathway (or JNK pathway) results in the activation JNK by the action of small GTPases. (C) The Ca^{2+} pathway is activated by PI-dependent release of intracellular calcium, which regulates the activity of enzymes such as PKC and CamKII and leads to the modulation of gene expression in the nucleus. Reproduced from reference (63) with permission from Elsevier © 2008.



the proteosome. Engagement of Fz by the Wnt ligand results in the inhibition of GSK3β and leads to the hypophosphorylation of β-catenin which accumulates in the cytoplasm and is translocated to the nucleus (reviewed in 153). In the absence of nuclear β-catenin, the T-cell specific transcription factor/lymphoid enhancer binding factor 1 (TCF/LEF) family of transcription factors binds DNA, along with Groucho and histone deacetylases (HDACs) to form a repressive complex. This complex, via the action of chromatin remodeling, blocks transcription of Wnt target genes (39, 44). Once in the nucleus, β-catenin replaces Groucho and converts the group of proteins into an activation complex which includes CBP/p300 and the chromatin remodeling enzyme Brg1 (81, 202). This complex, containing TCF/LEF and β-catenin can now induce the transcription of Wnt target genes (28, 137). Wnt signalling may also be transmitted in a β-catenin-independent fashion, often referred to as non-canonical Wnt pathways. One of these pathways, the Wnt/Ca^{2+} pathway, is thought to function through the release of intracellular Ca^{2+}, which activates Ca^{2+} sensitive enzymes such as Ca^{2+}-calmodulin-dependent protein kinase II (CamKII) and PKC (108, 191, 196). It has been proposed that the release of Ca^{2+} is dependent on G-protein linked phosphotidylinositol (PI) signaling (108, 191, 196). The second of these non-canonical pathways is often referred to as the planar cell polarity (PCP) pathway, which is involved in controlling cell polarity, apoptosis and asymmetrical cell divisions via the activation of JNK (22, 117) It has been proposed that the activation of JNK in this pathway is mediated by the Rho family of GTPases (14).

1.3.2.2 – Wnt signalling and mesodermal patterning

The expression profile of Frz receptors and Wnt ligands have been extensively characterized during embryogenesis, particularly in tissues involved in the formation of skeletal muscle. Fz1, Fz2, Fz6, Fz7 are expressed in newly formed somites, whereas Fz9 is only detectable at later stages in the myotome of E10.5 embryos (17, 38), implying a role for Wnt signalling during muscle development. In addition, mice harboring a spontaneous mutation in Lrp6, a Wnt co-receptor, display defects in the establishment of somite compartments, in somite epithelialization and in the formation of somite segmental borders (107). Wnt1,-3a and -4 are expressed in the dorsal half of the neural tube as epaxial myogenesis begins, which is consistent with the idea that known myogenic signals are derived from the axial midline structures (38, 86, 143, 156, 164). Mice null for Wnt3a lack caudal somites, have a disrupted notochord and fail to form a tailbud (229). Thus, it appears as though Wnt signalling is required very early on to regulate the fate of the paraxial mesoderm. Consistent with this, implantation of Wnt1,-3a,-4-secreting cells in the chick embryo enhances the expression of Paraxis, a gene required for somites to undergo epithelialization (32, 211). It has been proposed that Wnt3a acts to establish a gradient which controls segmentation of the PSM (3, 174). Knockout of Wnt3a in mice reveals that canonical Wnt signalling is involved in regulating the segmental clock by controlling the oscillation of Axin2 and lunatic fringe (Lnfg). Both Axin2 and Lnfg oscillations are disturbed in Wnt3a mutants, whereas the control of Axin2 expression is undisturbed in Notch signalling mutants, suggesting that Wnt signalling acts upstream of Notch-regulated cyclic genes (3, 163). β-catenin has also been shown to directly regulate Dll1, a Notch ligand, linking the Wnt and Notch signalling pathways together (61, 85).

1.3.2.3 – Wnt signalling and the establishment of myogenic identity

In addition to its role in specifying paraxial mesoderm cell fate, Wnt signalling also contributes to the patterning of the somite compartments. Ectopic expression in chick of Wnt1,-3a,-4, or the downstream effector activated β-catenin, results in the expansion of the epaxial muscle domain at the expense of the sclerotome, and expression of the DM markers Pax3 and Pax7 (37, 57, 155, 211). Mice null for both Wnt1 and Wnt3a show a loss of the medial compartment of the dermomyotome and reduction of Pax3 and Myf5 expression (91). Wnt6 can replace the dorsal ectoderm, a known source of pro-myogenic signal, and regulate the expression of Pax3 and Pax7 in the developing somite (155). The expression of other somite factors, such as Gli2, is also regulated by Wnt signalling. Studies in quail reveal that β-catenin activates somite-specific expression of Gli2, thus providing a link between the Wnt signalling pathway and the Shh pathway, both of which are responsible for Gli2 expression during somite patterning (18).

Wnt signals have also been implicated in the regulation of MRF expression during embryogenesis. Paraxial mesoderm explant studies reveal that Wnt1 signalling from the dorsal neural tube preferentially activates Myf5 expression, while Wnt6/7a signals from the dorsal ectoderm activate MyoD, suggesting that epaxial and hypaxial myogenesis can be differentially regulated by Wnt signalling (199). Blocking Wnt signals in the somite results in decreased Myf5 expression in the muscle progenitors of the epaxial domain, whereas the introduction of an activated β-catenin is sufficient to upregulate Myf5 expression in the somites (16). The spatiotemporal expression of Myf5 in the epaxial somite is dependent on the presence of TCF/LEF sequences in the 5' Myf5 early epaxial enhancer (16). So far, the majority of studies have focused on exploring the role of the canonical Wnt signalling

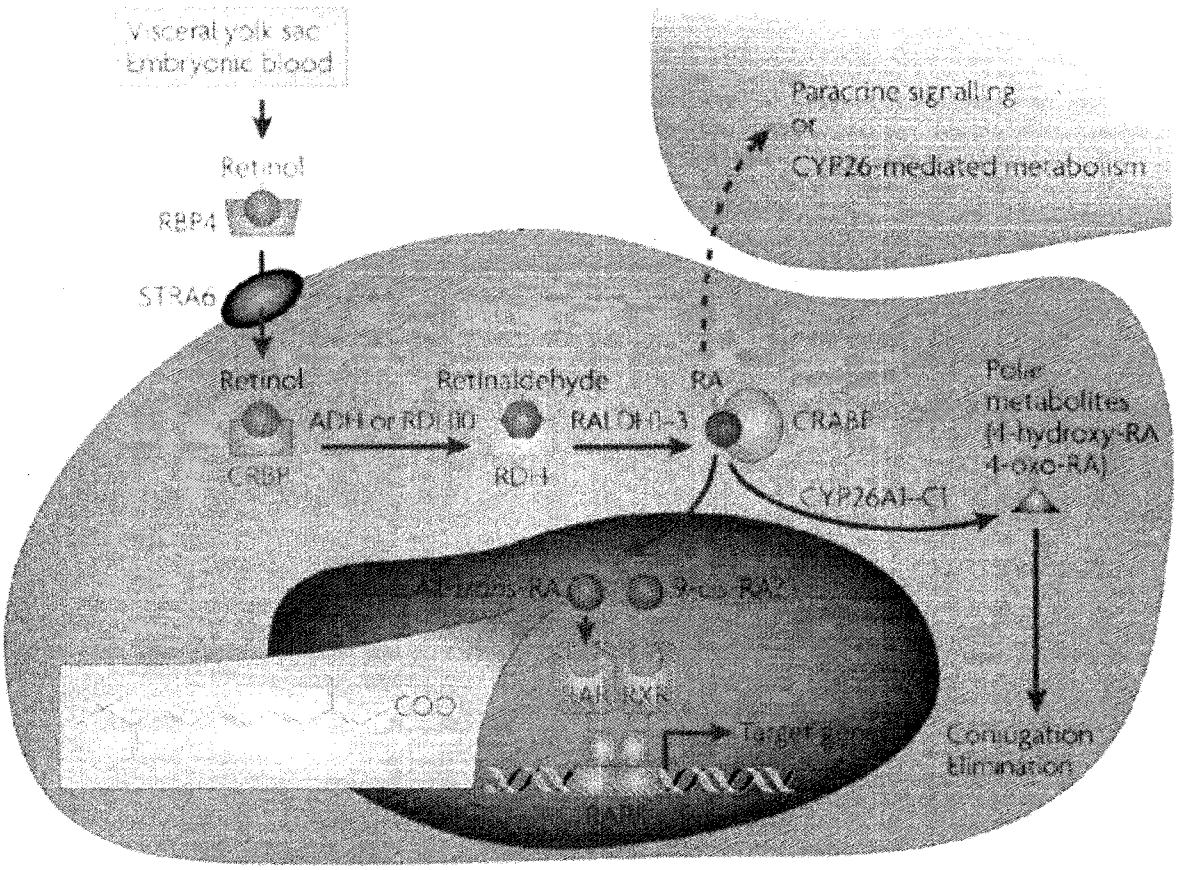
pathway in regulating the process of embryonic muscle development. Recently, two studies have identified β-catenin independent pathways of Wnt-directed myogenic gene expression. In PSM explants, modulation of Pax3 expression by PKC leads to the expression of MyoD, even in the absence of activated β-catenin and Myf5 (27). Adenylyl cyclase (AC) signalling via protein kinase A (PKA) and its downstream effector CREB has also been shown to mediate the activation of Myf5 and MyoD by Wnt1/7a (43).

1.3.3 – Retinoic acid

1.3.3.1 – RA Synthesis and Metabolism

Retinoic acid (RA), the active derivative of Vitamin A, is needed to regulate major embryonic growth and patterning decisions (reviewed in 150). Retinol, which is supplied maternally during embryogenesis, is taken up by the retinol binding protein 4 (RBP4), which is expressed from the pre-gastrulation stage. RBP4 then binds the cell-surface receptor Stra6, which was very recently identified in a screen for RA-regulated genes (104). Once inside the cell, RA synthesis from retinol, which is bound to the cellular retinol binding protein (CRBP) is under the control of the canonical pathway for RA synthesis, which is comprised of two steps (Figure 1.6). During the first step of synthesis, retinol is converted to retinaldehyde by the Alcohol Dehydrogenase (ADH) and Retinol Dehydrogenase (RDH) families of enzymes. Gene knockout studies have shown that the absence of RDH10 mimics the phenotypes observed in cases of RA-deficiency (33, 184). Low levels of RA synthesis are still detected in these tissues, suggesting that other enzymes, such as ADH7, can contribute to the regulation of RA synthesis (139, 184). The final step in the pathway

Figure 1.6 – RA synthesis: a two-step process. Circulating retinol is bound by the retinol-binding protein RBP4, which interacts with the retinol receptor Stra6, and leads to the internalization of retinol. Once inside the cell, retinol undergoes conversion to retinaldehyde by the action of members of the ADH or RDH family of enzymes. The next step in RA production involves the oxidation or retinaldehyde to retinoic acid, mainly through the action of the RALDH class of enzymes. RA is now free to bind its receptor, and translocate to the nucleus where it regulates gene expression. Reproduced from reference (150) with permission from Macmillan Publishers Ltd © 2008.



involves the oxidation of retinaldehyde to RA, which is mediated by the Retinaldehyde dehydrogenase (Raldh) class of enzymes. There are three Raldh family members, Raldh1,-2,-3. Raldh2, the first to be expressed, can be detected in the primitive streak, the mesodermal cells during gastrulation, and later becomes restricted to the posterior embryonic trunk region. Raldh2 is also detectable at the future cervical and trunk levels following differentiation (151). Mice which carry null alleles for Raldh2 die pre- and peri-natally and display defect affecting the forebrain, hindbrain, heart, limbs and somites (132, 133, 152, 170, 193, 206). Examination of Raldh1 and Raldh3 knockout mice suggests that these two enzymes function at later stages of embryogenesis, mainly during eye and nasal development (54, 127, 140).

RA metabolism represents an additional level of control for regulating local RA action. The three members of the cytochrome P450 26 A (CYP26A) enzymes, Cyp26a1,-b1 and –c1 are expressed in a wide variety of embryonic tissues, including the primitive streak and posterior mesoderm (Cyp26a1), the hindbrain (Cyp26b1-c1) and the limb buds (Cyp26b1) (119, 198). It has been proposed that the main role of the *CYP26A* gene products is to remove bioactive RA from tissues by metabolizing RA into the derivatives 4-OH RA and 4-oxo RA, thus regulating RA signalling (149, 220). In support of this hypothesis, mice lacking functional Cyp26a1 and Cyp26b1 display developmental abnormalities that phenocopy those of excess RA exposure (1, 182, 227).

1.3.3.2 – Mechanisms of RA signalling

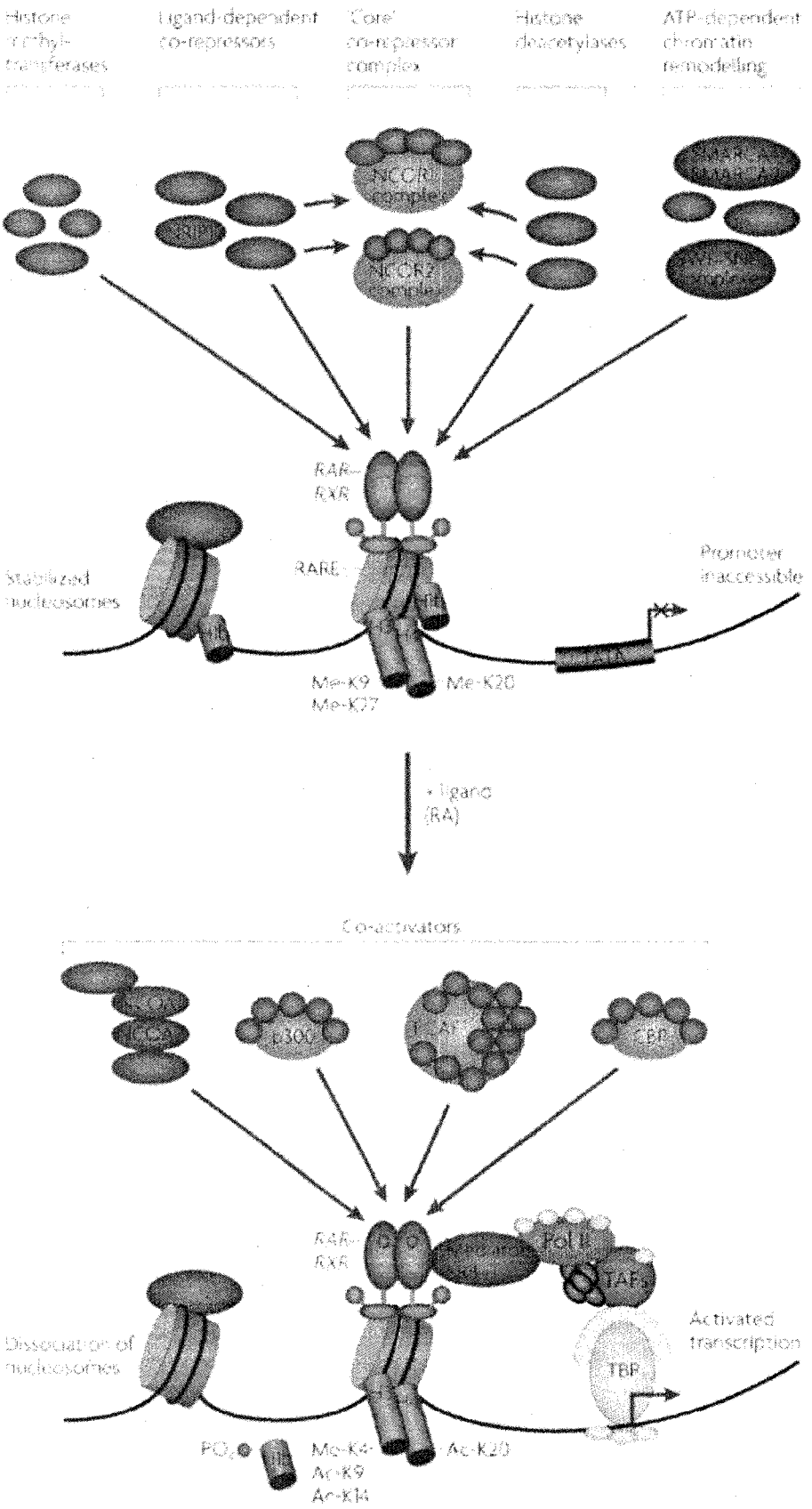
RA exerts its signalling effects by binding to the retinoic acid receptor (RAR), which exists in three types (α,β,γ). The RARs are ligand-inducible transcriptional activators which act as heterodimers with the retinoid X receptors (RXR), of which there are three types as

well (α,β,γ) (42, 65, 72, 121, 138). RAR/RXR heterodimers normally bind to retinoic acid response elements (RAREs) in the absence of ligand, and recruit co-repressors (Figure 1.7). These co-repressors mediate their effects by recruiting HDACs and methyl-transferase complexes to the DNA, so that it becomes inaccessible for transcription (45, 88, 89). RA binding to RAR induces a conformational change in the RAR-ligand binding domain which results in the release to co-repressors and the recruitment of transcriptional activators (112, 114, 209, 219). There have been hundreds of genes reported to be induced by RA, either directly or indirectly. This diverse repertoire of target genes nicely demonstrate the broad spectrum of effects exerted by RA during embryogenesis and underscore the extent of pleiotropic roles it adopts *in vivo*. In the following section, we will discuss in further details the role of RA in regulating mesodermal patterning.

1.3.3.3 – RA and mesodermal patterning

Due to the availability of model organisms in which RA synthesis has been pharmacologically or genetically inhibited, it has become clear that RA plays an important role in regulating segmental patterning during embryogenesis, particularly in establishing the bilateral symmetry of somite pairs. Raldh2, expressed in the rostral presomitic mesoderm, is expressed in an antagonistic pattern to Fgf8, which is detected in a caudal to rostral gradient (181, 206). It has been proposed that the functional antagonism between Fgf8 and RA establishes the proper signals for body axis extension (181). Loss of RA signalling leads to the formation of only a few somite pairs before somitogenesis proceeds in an uncoordinated manner along the left and right sides, resulting in one side with fewer somites than the other (105, 193, 206, 207). This is thought to occur because of the anteriorization of the FGF8 caudal gradient, as well as the asymmetric expression of Hes7 and Lnfg which are required

Figure 1.7 – Mechanisms of RA action. In the absence of ligand, the RAR/RXR heterodimer binds to the RAREs within the promoter/enhancer regions of target genes. Unliganded RARs recruit co-repressors which mediate their negative transcriptional effects by recruiting chromatin remodeling enzymes such as methyltransferases and histone deacetylases, which render the chromatin inaccessible for transcription. Binding of RA causes a conformational change in the RAR ligand-binding domain which results in the release of co-repressors and the recruitment of PCAF and p300. These co-activators induce changes in the local chromatin architecture which now renders it accessible to the basal transcriptional machinery, thus allowing the expression of RA target genes. Reproduced from reference (150) with permission from Macmillan Publishers Ltd © 2008.



to control the oscillating cycles of Notch expression that set up the somite boundaries (105, 206, 207). Therefore, RA appears to act as a buffer to prevent left-right asymmetry from occurring in the pre-somitic mesoderm, maintaining the bilateral symmetry observed during somitogenesis in mouse, chick and zebrafish.

1.3.3.4 – RA and the myogenic identity

Once the identity of the somitic compartments have been established, RA is still required for the regulation of genes involved in myogenic differentiation. Inhibition of RA synthesis in zebrafish using the chemical inhibitor DEAB results in a reduction of MyoD, Myogenin and fast myosin expression in somites. The addition of exogenous RA from the gastrulation stage onwards increased expression of these three genes in the somites and induced premature expression of MyoD and Myf5 in the PSM (77). Furthermore, RA is able to enhance MyoD expression and induce myogenic differentiation in C2C12 myoblasts, cultured chick satellite cells and limb buds (2, 76, 141).

1.4 – P19 CELLS: A MODEL OF MYOGENIC DIFFERENTIATION

Genetic analysis of animal models carrying gain-of-function or loss-of-function mutations has been the main source of experimental evidence in the search to elucidate the mechanisms which regulate early embryonic development. However, due to the limited amount of material available, it is often difficult to study the events involved in the very early steps of development. As such, embryonal carcinoma (EC) cell lines have proven an invaluable tool to scientists, given that large amounts of material can be obtained for analysis and that they are relatively amenable to genetic manipulations.

P19 cells are a stable, euploid EC cell line, which was originally derived by grafting an E7.0 embryo into the testis of a male mouse to induce teratocarcinoma formation. After several months, the primary tumours, which usually contain a mixture of differentiated and undifferentiated cell types, were harvested and undifferentiated cells from one of these tumours was used to derive the P19 EC line (129). Cultured P19 cells are able to maintain their pluripotency following propagation in culture. Aggregated P19 cells were injected into the inner cell mass of early-stage embryos in order to generate chimeric blastocysts. P19 cells could be detected in all tissues analyzed, including the liver, gut, brain, heart, lung, spleen, blood and muscle, indicating that P19 cells are able to contribute to the normal development of tissues derived from all three embryonic germ layers (177). These embryos, however, did show signs of abnormal morphology, including large P19-cell derived tumours in the head region (177).

P19 cells can be induced to differentiate into skeletal muscle by aggregation in plastic dishes for four days in the presence of DMSO, followed by transfer to tissue-culture grade dishes for an additional 5 days (179). In order for differentiation to take place, P19 cells require both aggregation and drug-treatment, as cells grown in monolayer and exposed to DMSO fail to form skeletal myocytes (128). Exposure to concentrations of DMSO in the range of 0.5-1% results in approximately 5-15% of total cells which are MHC-positive skeletal myocytes, while neuronal cell types are not detected under these culture conditions (56, 128, 179). The mechanisms by which exposure to DMSO triggers skeletal myogenesis are not fully understood. DMSO has been demonstrated to control the release of Ca^{2+} from intracellular stores, as well as contributing to the epigenetic modification at multiple loci throughout the genome (93, 142). Unidentified factors in fetal calf serum preparations have

also been shown to affect the extent and efficiency of P19 cell differentiation into muscle (224).

Skeletal myocytes produced from differentiated P19 cells are physiologically similar to embryonic tissue, such that both cell types express isoforms of muscle sarcomeric proteins, including MHC and cardiac alpha-actin as well as the MRFs (194, 195). The following section aims to provide an overview of the advances made in elucidating the regulatory mechanisms governing muscle development using P19 EC cells.

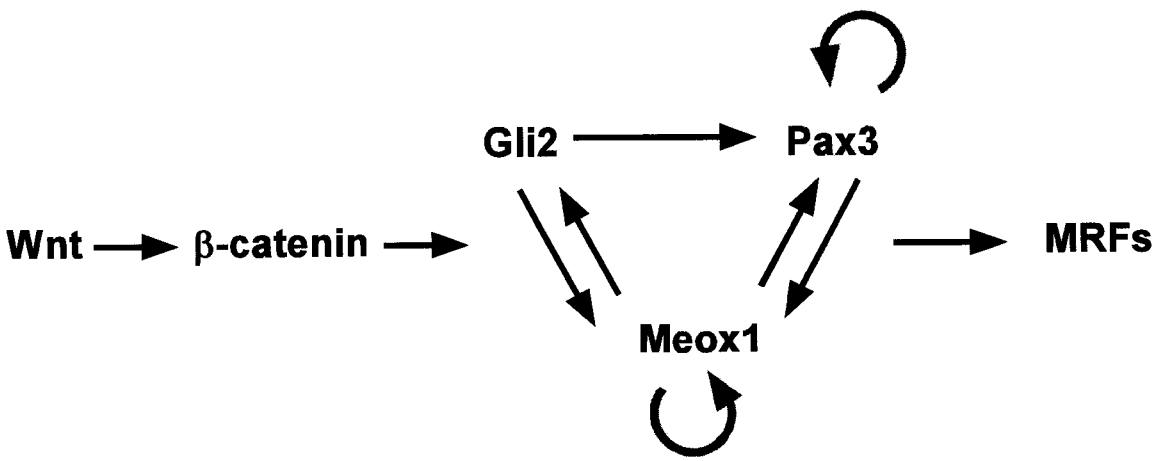
The temporal expression patterns of genes examined to date in differentiating P19 cells recapitulates the order of expression observed in the mouse embryo, beginning with the onset of Brachyury-T (T) expression, which is involved in mesoderm formation (8). As embryonic development progresses, the mesoderm is further patterned into somites, which serves as the source of muscle precursor cells (reviewed in 29). During DMSO-induced differentiation of P19 cells, T expression (208), which can be detected as early as day 1, is followed by the onset of Pax3, Meox1, Gli2, Six1 and Eya1 (171, 172), transcription factors which are expressed in the dermomyotome of the somite (reviewed in (31). Finally, a few days later, we can detect MyoD, Myf5 and Myogenin expression (171, 172). Thus, P19 cells are able to provide information about the regulatory mechanisms controlling embryonic development, since they are able to recapitulate the myogenic gene expression program *in vitro*. Further understanding of the function and regulation of these gene products has also been determined in P19 cells.

Several series of gain-of-function and loss-of-function experiments were performed by targeting multiple genes known to be involved in myogenesis. Cells stably expressing either Wnt3a, P19[Wnt3a], or an activated form of its downstream effector β-catenin have been generated and characterized (160). Ectopic expression of either Wnt3a or the activated

form of β-catenin is sufficient to induce the expression of Pax3, Meox1 and Gli2, as well as MyoD and MHC. Conversely, a dominant-negative form of β-catenin abolishes expression of these genes and prevents myogenesis from occurring, suggesting an important role for β-catenin in regulating P19 cell myogenesis (160). This approach has also been used to identify the existence of small network regulatory motifs, such as feed-forward and autoregulatory loops. Examination of the phenotype of P19 cell lines which stably express Gli2, Pax3 or Meox1, as well as their dominant-negative counterparts, revealed that the three genes act together in a regulatory loop to induce each other's expression. Gli2 is both sufficient and necessary for Pax3 and Meox1 expression, while ectopic expression of Meox1 is sufficient to regulate the expression of Pax3 and Gli2 (159). Furthermore, Pax3 is able to induce the expression of Meox1 (172). Taken together, these observations suggest that all three genes exert their function by regulating each other's expression, revealing yet another layer of complexity in transcriptional regulation of P19 cell differentiation. These analyses have also revealed that Pax3 and Meox1 are able to autoregulate their own expression, as endogenous transcripts for these two genes were lost in the presence of dominant-negative mutants (160, 172). MRF expression can also be modulated by the presence or absence of functional Pax3, Meox1 and Gli2. MRF and MHC expression was lost in the presence of dominant-negative mutants of Pax3, Meox1 and Gli2, whereas only Pax3 and Gli2 were sufficient to induce myocyte differentiation in the absence of DMSO (160, 172) (Figure 1.8).

P19 cells have also been a useful model to study the effect of signalling molecules on the process of differentiation. In these cells, the expression of a Wnt3a construct triggers the specification of cells to the myogenic lineage, and subsequent differentiation into bipolar skeletal myocytes (160). Wnt3a also leads to activation of MRF

Figure 1.8 – Transcriptional regulation of Pax3, Meox1 and Gli2 during skeletal myogenesis in P19 cells. Using gain-of-function and loss-of-function studies, it was discovered that Gli2, Meox1 and Pax3 act in a feed-forward loop, regulating each other's expression. Also, Pax3 and Meox1 are able to auto-regulate during myogenesis. These observations underscore the complexity of the gene regulatory motifs which regulate differentiation in P19 cells. Reproduced from reference (159) with permission from ASBMB Journals © 2004.



function which causes an increase in the number of MHC-positive cells in P19[MyoD] cell lines (171). A role has also been identified for Sonic Hedgehog (Shh) in controlling cardiac myogenesis in P19 cells, by inducing the expression of Nkx2.5, GATA4 and Mef2c (64). P19 cells constitutively express the retinoic receptors RARα and RARγ, while RARβ transcript and protein levels are upregulated during DMSO-induced differentiation into muscle. The observed change in RARβ levels is associated with an increased sensitivity to RA (165). Consistent with this observation, RA has been demonstrated to both positively regulate skeletal myogenesis while inhibiting cardiac muscle development in P19 cells (Kennedy and Skerjanc, unpublished results). In this case we see acceleration/enhancement of Pax3, Meox1, Sox7 and MRF expression, consistent with the phenotype of precocious differentiation observed during embryogenesis (77).

1.5 – Myogenesis in Mouse and Human Embryonic Stem Cells

Although some of the molecular events regulating skeletal muscle precursor determination and differentiation in P19 embryonal carcinoma cells have been extensively studied, not much progress has been made in elucidating the networks which control differentiation in mouse (mES) and human (hES) embryonic stem cells. Stem cells are characterized by their ability to self- renew, proliferate and retain the capacity to differentiate into multiple different cell types. Although mES cells have been available to scientists from the last 20 years, they have not been studied extensively for their ability to differentiate into muscle. This may be due to the relative difficulty of obtaining sufficient amounts of skeletal muscle *in vitro*, compared to the ability to examine myogenesis in knockout animal embryos. Nevertheless, early studies have shown that the *in vitro* differentiation of mES cells is able to

recapitulate the early steps of mammalian muscle development, which includes the expression of the MRFs and calcium channels (176). Several reports have reported the successful differentiation of mES and hES cells into muscle in culture (7, 175, 176, 231). ES-cell derived muscle responds in a similar fashion as embryonic muscle to cues which pattern the mesoderm and regulate differentiation. mES cells expressing m-Twist display delayed differentiation, accompanied by a decrease in MRF expression, which is similar to the phenotype observed in mice (175, 197). Recent studies have focused on isolating paraxial mesoderm progenitors or skeletal muscle precursors induced from ES cells followed by engraftment into muscle tissue in order to assess the differentiation potential of these purified cell populations. It has been reported that ES cells injected directly into the muscles of mice do not undergo significant or preferential myogenic differentiation, suggesting that the alteration of gene expression induced by aggregation may be required in order for differentiation to take place under these circumstances (205). The EB system typically used for ES cell differentiation does not recapitulate the formation of the notochord or neural tube, which are sources of inductive cues for paraxial mesoderm patterning. To circumvent this deficiency, an ES cell line was engineered to express an inducible form of Pax3. As discussed in section 1.2.2, Pax3 is expressed in the muscle progenitor cells of the embryo. Following purification and injection of these Pax3-positive cells into the muscles of mice, significant engraftment and functional improvement of the impaired muscle could be observed (49). The enrichment of mesodermal and myogenic progenitors from differentiating ES cells prior to engraftment has proven, in several studies, a successful strategy for the regeneration and repair of dystrophic muscle tissue (6, 11, 183). The transplantation of these more primitive cells may prove more advantageous given their high replicative potential (183).

1.6 – Thesis Summary

The mechanisms which control the cell's decision to proliferate or differentiate during embryonic development are a very complex series of events that ultimately generate the fully differentiated tissues that make up an organism. Being able to control a stem cell's decision to differentiate into a given tissue has enormous therapeutic potential for a wide variety of human disorders. However, in order for stem cell therapy to become reality, scientists must have a sound understanding of the networks and molecules which regulate the entire process of muscle development. The initial goal of this research project was to identify factors upstream of Pax3, a marker of the muscle precursor population. The rationale behind this thinking was that if molecules that can enhance Pax3 expression could be identified *in vitro*, cultures could be manipulated in order to enhance the number of cells which ultimately enter the myogenic lineage.

In Chapter 2, the experiments were designed based on the hypothesis that Sox15, which is expressed in the myoblast, but not the myocyte, plays a role in maintaining the undifferentiated, but committed, phenotype by regulating Pax3 expression. Here we present data that Sox15 enhances expression of muscle precursor genes, but not that of the MRFs or MHC. Furthermore, loss of Sox15 expression/function leads to downregulation of Pax3 and Meox1. We go on to demonstrate the Sox7 transcripts are not upregulated by Sox15, and that Sox15 can bind regions upstream of Sox7, raising the possibility that Sox15 may negatively regulate Sox7 during skeletal myogenesis in P19 cells.

In Chapter 3, experiments were performed in order to test the hypothesis that signalling pathways that are active during myogenesis, particularly the Wnt and Shh pathways, regulate the expression of the pre-skeletal mesoderm markers Foxc1 and Foxc2. Using approaches that both positively and negatively regulate these pathwas, we have

established that both Wnt and Shh signalling pathways contribute to the expression of Foxc1 and Foxc2 transcripts during myogenesis in P19 cells.

In Chapter 4, the objective was to further extend the role of Sox7 during muscle development by exploring the pathways upstream that are required for its proper expression. Based on our observations, we propose that both the RA and Wnt signalling pathways contribute to Sox7 expression. The studies presented here further extend our knowledge of the molecular networks of pathways and transcription factors which control the process of myogenic differentiation in P19 cells.

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Chapter 2

Sox15 and Sox7 Differentially Regulate the Myogenic Program in P19 cells[1]

[1]A version of this chapter has been submitted

Savage, J., Conley, A.J., Blais, A., and Skerjanc, I.S., (2008) Sox15 and Sox7 Differentially Regulate the Myogenic Program in P19 Cells. *In revision with Stem Cells*

2.1 - INTRODUCTION

During the process of vertebrate skeletal myogenesis, signalling molecules such as Wnts, Bone Morphogenetic Proteins (BMPs) and Sonic Hedgehog (Shh) are secreted from tissues surrounding the somite (10, 11, 40). These extracellular cues activate a cascade of transcription factors that ultimately leads to the expression of the myogenic regulatory factors (MRFs) MyoD, Myf5, Myogenin and MRF4, which control the end-stages of myogenesis (1). Induction of MRF expression in response to these signals appears to be mediated by many transcription factors including Pax3, Meox1/2, Six1 and Gli2 (5, 6, 17, 22, 23, 29, 31, 36).

Pax3, Meox1 and Gli2 are part of a regulatory network of transcription factors that regulate myogenesis (29, 36). Pax3 is expressed in the mediolateral dermomyotome and migrating limb precursors (13, 46), while Pax7, a closely related member, is also expressed in the dermomyotome and may have redundant functions with Pax3 (32). Cell culture studies reveal a role for Pax3 in regulating the commitment of cells to the myogenic lineage. Ectopic expression of Pax3 in embryonic tissues is sufficient to activate MyoD and Myf5 expression (23, 34), and overexpression in P19 cells also leads to the induction of myogenesis and MRF expression (36). Recent identification of a Pax3 binding site within the Myf5 regulatory region suggests that Pax3 can directly activate Myf5 expression in developing murine limbs (2). Furthermore, P19 cells expressing a dominant-negative form of Pax3, termed Pax/EnR, are unable to undergo myogenesis and do not express MyoD (36). Expression of Pax3/EnR and Pax7/EnR also inhibits MyoD expression in the mouse embryo (31), and animals lacking both Pax3 and Pax7 form myotomal muscle, but do not continue to develop primary or secondary muscle fibers (33). Pax3/Pax7 are essential for the formation

of satellite cells, suggesting that the Pax3/Pax7 pool of progenitor cells is responsible for nearly all skeletal muscle formation (14, 33).

A role for Meox1 in regulating skeletal myogenesis and Pax3 expression has been demonstrated (22, 29). Meox1 and Meox2 are two closely related homeobox transcription factors. Both Meox1 and Meox2 are initially expressed in the somites, and their expression becomes localized to the dermomyotome and the developing limb bud respectively, as development proceeds (6, 7). Mice carrying null mutations for both Meox genes show a loss of Pax3 and Pax7, in the somite (22). These results place Meox1 prior to Pax transcription factors in the molecular hierarchy controlling myogenesis. Consistent with this, a dominant-negative Meox1 transcription factor, Meox1/EnR, downregulates Pax3 expression and ablates myogenesis (29). It has been shown, by our lab as well as others, that Wnt signalling can activate Pax3 expression, although it is unclear whether this activation is direct or indirect (8, 12, 30, 45). The transcription factor Gli2 is also able to induce Pax3 expression, while ectopic expression of a Gli2 dominant-negative, Gli2/EnR, inhibits Pax3 expression and ablates skeletal myogenesis (29). Although much is known about the downstream effects of Pax3, Gli2 and Meox1 during myogenesis, less is understood about the factors that regulate their gene expresion.

Sox transcription factors have been characterized for their involvement in muscle development. Sox8 has been identified as a marker of adult satellite stem cells, and has the ability to inhibit differentiation of cultured primary myoblasts when ectopically expressed (37). Studies have implicated another factor, Sox15, as a regulator of myogenesis. Murine Sox15 was cloned using a differential gene expression screen between proliferating and differentiating myoblasts (4). Sox15, when ectopically expressed in C2C12 myoblasts, was able to block myotube formation (4). Moreover, disruption of Sox15 in mice resulted in

attenuation of skeletal muscle regeneration following crush injury accompanied by a decrease in MyoD expression (20). It is believed that Sox15, along with Fhl3, binds and regulates Foxk1 expression in regenerating muscle (24). Sox7, another member of the Sox family, was first characterized in Xenopus (38), followed by the identification of homologs in mice (41, 42) and humans (16). Sox7 is detected in the somite of mice as early as embryonic day 7.5-8.0 (41). Studies in Xenopus have shown that xSox7 can induce the expression of mesoderm inducing genes Xnr1-6 and Mixer, suggesting a role for Sox7 in regulating the cell fate of mesodermal cells (49). In addition, Sox7 has been extensively characterized for its role in controlling arteriovenous specification in zebrafish (9, 15, 27) and Xenopus (50).

Based on the evidence in the literature that Sox15 and Sox7 are involved in controlling the cell fate of mesodermal derivatives, the present study aims to further characterize the role of SOX transcription factors in regulating the expression of skeletal muscle precursor genes during differentiation in P19 cells.

2.2 - Materials and Methods

2.2.1 - DNA Constructs

Expression constructs of PGK-pPuro, PGK-Lacz, B17, PGK-Pax3, PGK-Meox1, CMV-Gli2 and activated CMV-β-catenin have been previously described (29, 30, 35, 36). CMV-Sox15 was kindly provided by F. Béranger (4). PGK-Sox7 was created by excision of the Sox7 ORF from the pCMVScript vector (kind gift from Y. Hayashi, Japanese Science and Technology Agency). The ends of the excised insert were blunted in order to form

compatible ends for cloning into the SmaI site of the PGK vector, which has been previously described (39).

The dominant-negative Sox15/EnR fusion protein was created by PCR amplification of the *engrailed* (EN-2) repressor domain using the following oligonucleotides: EnR-F 5'-AACTCGAGAGAGGAGAAGGATTCCAAGCCC and EnR-R 5'-TTGAATTCCTAGCCCAGAGTGGCGCTGGCTT. Restriction sites for *XhoI* and *EcoRI* were introduced for cloning purposes and are underlined in the sequence. The activation domain of Sox15 was removed by digesting the pRK5-Sox15 vector using the restriction enzymes *XhoI* and *EcoRI* (removes nucleotides 376 to 696 from the Sox15 cDNA) and replaced by ligation with the *engrailed* repressor domain. DNA sequencing was performed to ensure that the resulting chimeric protein was in-frame.

2.2.2 Cell Culture and Transfections

P19 Embryonal Carcinoma (EC) cells were cultured as previously described (47) in α-minimal essential media (Invitrogen, Burlington, Canada) supplemented with 5% Cosmic Calf Serum (Hyclone, Logan, UT) and 5% Fetal Bovine Serum (CanSera, Rexdale, ON, Canada). P19[Control], P19[Sox15], P19[Sox15/EnR], and P19[Sox7] cell lines were created as previously described (29, 30, 36). Cells were differentiated in the presence or absence of DMSO as previously described (35). RNA was harvested on the days indicated for Q-PCR analysis and cells were fixed for immunofluorescence. Seven clones were examined for the P19[Sox15] cell line, four for P19[Sox15/EnR], four for P19[Sox7], and four for P19[Control] cells. Little variability was observed between clonal populations for a given cell line.

2.2.3 – Immunofluorescence

Myosin Heavy Chain (MHC) expression was detected using the MF20 monoclonal antibody as previously described (35). A 1:100 dilution of goat anti-mouse Cy3-linked antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS was used as a secondary antibody. For Pax3 and Meox1 detection, coverslips were fixed at -20°C in acetone for 10 minutes prior to rehydration in PBS. Following permeabilization in 0.5% Triton X-100/PBS, cells were blocked using a 10% FBS, 0.1% BSA and 0.1% Triton X-100 solution in PBS at room temperature for 1 hour. The coverslips were incubated overnight using a 1:50 dilution of Meox1 antibody (sc-10185, Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:50 dilution of Pax3 antibody (clone 274212 , R&D Systems, Minneapolis, MN), followed by a 1 hour room temperature incubation with a 1:100 dilution of donkey anti-goat Cy3-linked antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS or a 1:100 dilution of goat anti-mouse Cy3-linked antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS, respectively. Immunofluorescence was detected using a Zeiss Axioscope microscope. Images were captured on a Sony 3CCD camera and processed with Axiovision, Adobe Photoshop7 and Canvas 8. For cell counting experiments, 10 fields of view per coverslip were counted using the 20x objective. Two coverslips for each treatment were counted, and the results shown are the average of three independent differentiations ± SEM.

2.2.4 - Reverse Transcription and Quantitative PCR

RNA was isolated using the RNeasy Mini Kit, according to the manufacturer's protocol. (Qiagen, Mississauga, ON, Canada). 1 μg of the purified RNA was used during the first strand DNA synthesis reaction using the Quantitect Reverse Transcription Kit, as per the

manufacturer's specifications (Qiagen, Mississauga, ON, Canada). For the PCR reaction, $1/20^{th}$ of the reverse transcription was used as a template for quantitative PCR amplification, which was carried out using the FastStart SYBR Green kit from Roche (Roche Applied Sciences, Laval, QC, Canada). All reactions were performed and analyzed using the ABI 7300 system and SDS analysis software (Applied Biosystems, Streetsville, ON, Canada). Primers used for Q-PCR analysis are listed in Supplementary Table 1.

2.2.5 - Microarray Analysis

For microarray analysis using the mouse genome 430 v2.0 Affymetrix array, P19[Sox15] and P19[Control] cells were differentiated in the absence of DMSO and RNA was extracted on day 5 using the Rneasy Mini Kit (Qiagen, Mississauga, Canada), according to the manufacturer's instructions. All procedures including labeling, hybridization and scanning were performed at the Ottawa Genome Centre (Ottawa, ON, Canada). Data analysis was performed with the dChIP program (21), using the PM-only method and filtering out probes with near-background expression levels in both samples. A probe representing the Sox15 mRNA was among the most induced genes in this analysis.

2.2.6 - RNA interference

Complementary DNA sequences targeting nucleotides 269 to 287 (5'-TTTGGATGAAGAGAAGCGACCCTTTCAAGAGAAGGGTCGCTTCTCTTCATCGCTTTTT-3' and 5'-CTAGAAAAAGCGATGAAGAGAAGCGACCCTTCTCTTGAAAGGGTCGCTTCTCTTCATC-3') and nucleotides 514 to 533 (5' TTTGCCTGGCAGTTACACCTCTTCTCAAGAGAAAGAGGTGTAACTG CCAGGCATTTTT -3' and 5'-CTAGAAAAATGCCTGGCAGTTACACCTCTTTCTCTTGAG

AAGAGGTGTAACTGCCAGG-3' of mouse Sox15 (NM_009235) were annealed and cloned into mU6pro using Bbsl and XbaI restriction enzymes. A control vector was created using a scrambled sequence that is not complementary to any sequences in the mouse genome (5'-TTTGGCTAAGCGAGCTGCTAGAGTTCAAGAGACTCTAGCAGCTCGCTTAGCTTTTT-3' and 5'-CTAGAAAAAGCTAAGCGAGCTGCTAGAGTCTCTTGAACTCTAGCAGCTCGCTTAGC-3'. The mU6pro vector was a generous gift from Dave Turner (University of Michigan, Ann Arbor, MI) and has previously been described (48). For the generation of stable cell lines expressing the short hairpin constructs, 0.8 μg of either shSox15 or shScrambled were transfected into P19 cells as previously described (35). Following puromycin selection, individual clones were either picked and expanded for further analysis or pooled together and further analyzed.

2.2.7 - Chromatin Immunoprecipitation

Five 150 mm dishes of day 5 P19[Sox15] aggregates were fixed using 1% formaldehyde at room temperature for 45 minutes, and quenched by adding 0.125M glycine. Cells were washed in ice-cold PBS and resuspended in lysis buffer 1 (50mM HEPES-KOH pH 7.4, 140mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100 and complete protease inhibitor cocktail (Roche Applied Sciences, Laval, QC) and incubated at 4°C for 10 minutes with rocking. Cells were then resuspended and incubated for an additional 10 minutes at room temperature in lysis buffer 2 (200mM NaCl, 1mM EDTA (pH 8.0), 0.5mM, EGTA (pH 8.0), 10mM Tris (pH 8.0) and protease inhibitor cocktail) prior to resuspension in sonication buffer (1mM EDTA (pH 8.0), 0.5mM EGTA (pH 8.0), 10mM Tris (pH 8.0) and protease inhibitors). Cells were sonicated using a Sonic Dismembrator (Fisher Scientific) for a total of 15 x 30 second pulses (1 minute rest between pulses) and lysates were cleared by centrifugation at 13,000rpm for 30 minutes at 4°C. The lysates were

pre-cleared by incubation with protein-G sepharose beads for 1 hour, and the chromatin was separated into three aliquots supplemented with 1% TritonX-100, 0.1% sodium deoxycholate, 1mM EDTA, 1mM AEBSF. An input sample of 1% of the total chromatin was set aside. For immunoprecipitation, 5 μg of Sox15 antibody (sc-17354, Santa Cruz Biotechnology, Santa Cruz, CA) or 5 μg of IgG antiserum (Zymed Laboratories, CA) was used. Following overnight incubation with the antibodies, the immune complexes were captured by addition of protein-G sepharose beads for 1 hour, followed by 10 minutes washed in each of the following buffers: low-salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA , 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high-salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl) and LiCl (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1mM EDTA, 10 mM Tris pH 8.1) and TE (10 mM Tris-HCl pH), 1 mM EDTA). Protein/DNA complexes were eluted using 50 mM Tris pH 8.0, 1 mM EDTA, 1% SDS, 50mM $NaHCO_3$ and crosslinks reversed overnight at 65°C by the addition of 200 mM NaCl. Samples were treated with 20 ug Rnase A and 40 ug Proteinase K and DNA was purified using Qiagen's PCR Purification Kit (Qiagen, Mississauga, ON). Relative enrichment of binding sites compared to the IgG negative control immunoprecipitation was analyzed using SYBR Green real-time PCR, as described above. Please refer to the supplementary information for a list of primers used in this experiment.

2.2.8 - Statistical Analysis

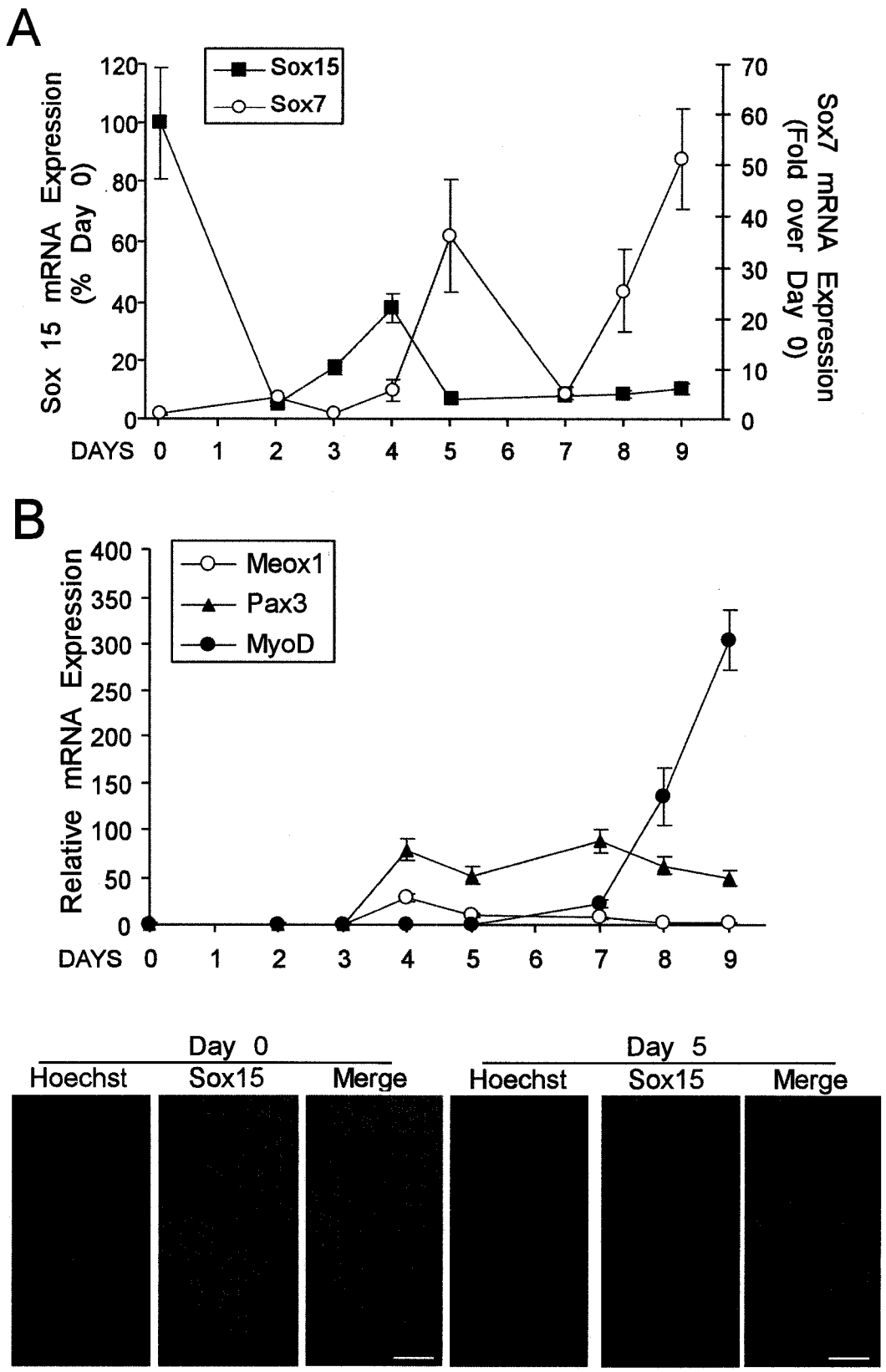
Statistical differences between means were calculated using either the Student's t-test or ANOVA. P-values of at least $p<0.05$ were considered significant.

2.3 RESULTS

2.3.1 - Sox15 expression is downregulated in a population of cells during myogenesis in P19 cells

In order to determine whether Sox15/Sox7 regulate the molecular program controlling muscle precursor cell formation, we sought to establish the temporal pattern of Sox15 and Sox7 mRNA expression in differentiating P19 cells by Q-PCR. Sox15 transcripts were highly abundant in undifferentiated stem cells and transcript levels decreased during differentiation, but were still detectable throughout the entire time course of differentiation and showed a small peak of expression on day 4 (Figure 2.1, A). Sox7 mRNA levels, on the other hand, showed a peak of expression of 36-fold (±11) on day 5 of differentiation, compared to day 0 (Figure 2.1, A). Sox7 showed maximal expression on day 9 of the time course. Both Sox7 and Sox15 were expressed on days 4 and 5, which correspond to peaks in Pax3 and Meox1 expression (Figure 2.1, B). This overlap in expression patterns indicate that Sox7/15 are expressed at a time which is consistent with their potential role in initiating the formation of muscle precursors during skeletal myogenesis in embryonal carcinoma cells. The presence of skeletal muscle in this experiment was confirmed by the detection of MyoD transcripts (Figure 2.1, B). To determine whether the decrease in Sox15 mRNA is a global decrease in transcript numbers in all cells, or a selective decrease in a subpopulation of cells, we performed localization studies using an antibody against Sox15. Immunofluorescence imaging indicated that Sox15 expression was detected in the majority of undifferentiated P19 cells, and that protein expression became restricted to a sub-population of cells as differentiation progressed (Figure 2.1, C).

Figure 2.1 – Sox15 expression is downregulated in a population of cells during myogenesis in P19 cells. Panels A and B: P19 cells were differentiated in the presence of DMSO and RNA was harvested daily for 9 days for analysis by quantitative PCR. The data was normalized to GAPDH, and results were expressed relative to day 0. Error bars represent average ± SEM, and the reactions were performed in triplicates. The experiment shown is representative of a typical differentiation. Panel C: The expression pattern of Sox15 protein on days 0 and 5 was confirmed by immunofluorescence using an anti-Sox15 antibody (red). Nuclei were stained by Hoechst (blue). Images are shown at 400x magnification and the scale bar equals 20μm.



2.3.2 - Sox15 upregulates the expression of muscle precursor genes

To ascertain the role of Sox15 during the early stages of myogenesis, we performed gain-of-function studies by isolating clonal populations of cells stably expressing Sox15, termed P19[Sox15]. To identify the transcription factors that may be regulated by Sox15, RNA from P19[Control] and P19[Sox15] clonal populations, aggregated without DMSO, was harvested on days 0 and 5 and subjected to quantitative gene expression analysis by Q-PCR (Figure 2.2.1, A). Sox15 was overexpressed an average of 79-fold (+/- 12, n=6) to 187-fold (+/- 71, n=6) over background (Figure 2.8, A). In the absence of DMSO, ectopic expression of Sox15 upregulated Pax3 and Meox1 transcript levels 92-fold (±66, n=5) and 546-fold (±310, n=5), respectively, compared to a 2-fold (±1, n=3) change in Pax3 expression and a 55-fold (±33, n=3) change in Meox1 expression observed in the P19[Control] cell lines (Figure 2.2.1, A). Additionally, we noted an 8-fold (±3, n=5) increase in mRNA expression for Pax7 and an 18-fold (±9, n=5) upregulation of Foxc1, both markers of pre-skeletal mesoderm (Figure 2.2.1, B). To differentiate between an increase in transcript copy number per cell and an increase in the number of muscle precursors actually formed, we performed immunofluorescent staining using antibodies against Pax3 and Meox1. Similar to the results obtained when examining mRNA expression levels, both Pax3 and Meox1 protein levels were upregulated in P19[Sox15] cells when compared to P19[Control] cells (Figure 2.2.2, A). Quantification of the Pax3 and Meox1 positive population revealed that 21% ± 2% (n=5) of the total cell population was Pax3 positive and 16 ± 3% (n=4) was Meox1 positive (Figure 2.2.2, B). These results suggest that expression of Sox15 is sufficient to enhance the proportion of cells specified to the myogenic lineage.

Figure 2.2.1 – Sox15 induces the mRNA expression of muscle precursor genes. P19[Control] and P19[Sox15] stable cell lines were differentiated in the absence of DMSO and analyzed for the expression of Pax3/7, Meox1 and Foxc1 using quantitative PCR. For this experiment, the data has been normalized to the expression of the housekeeping gene GAPDH, and is expressed relative to day 0 P19[Control] cells. Here, error bars represent average ± SEM, of 3 independent differentiations of 2 separate clones.

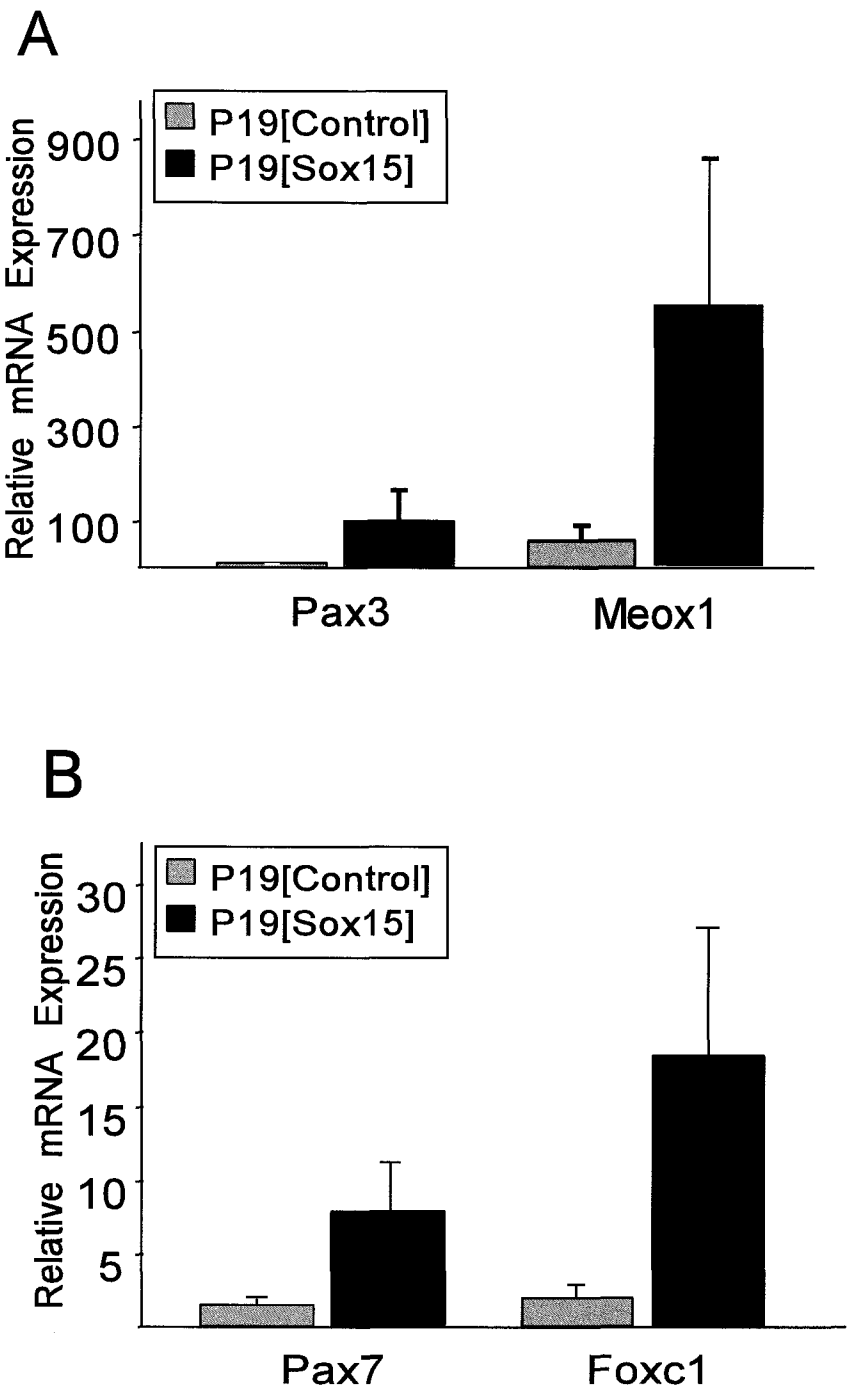
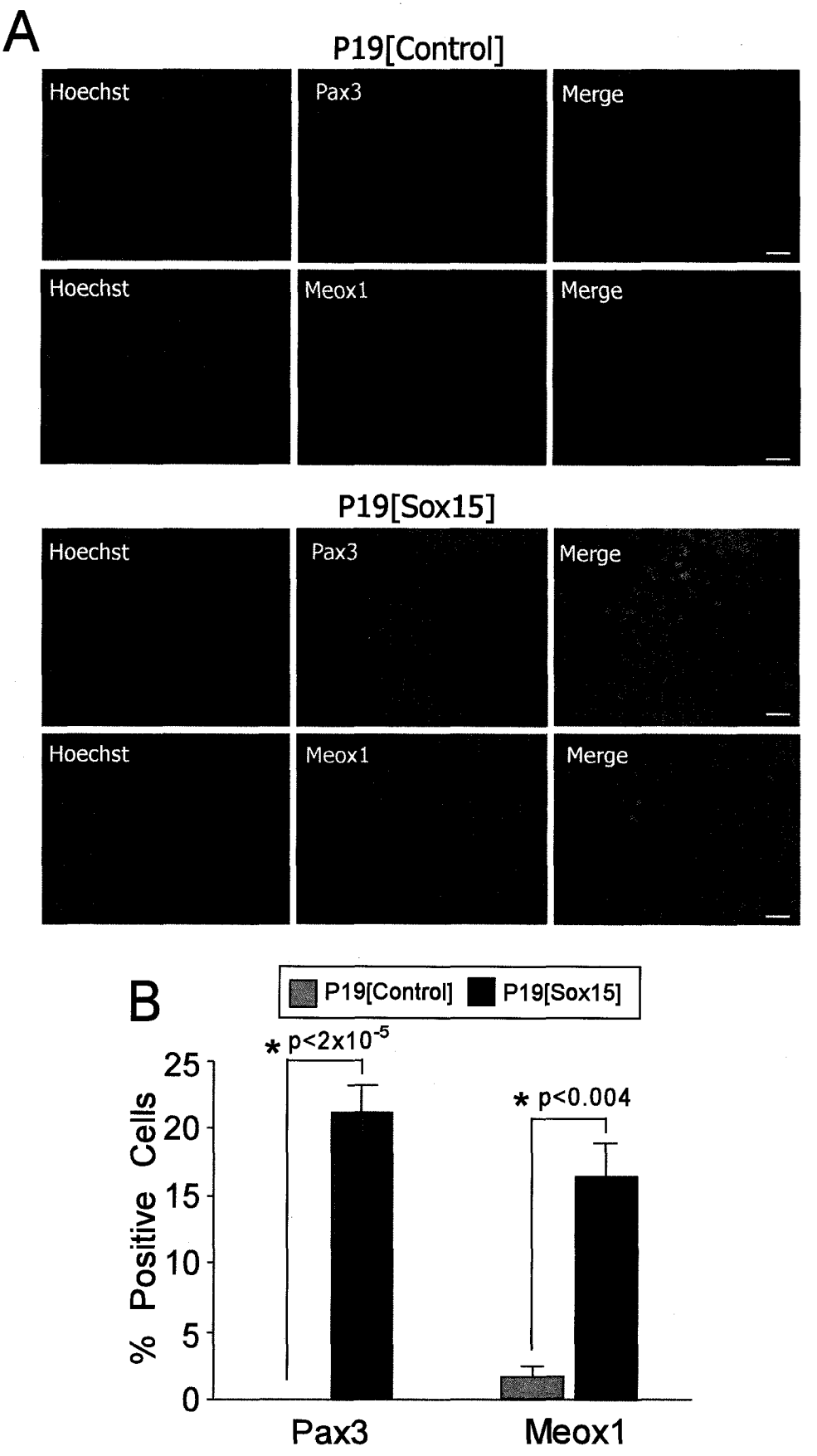


Figure 2.2.2 – Sox15 induces the protein expression of muscle precursor genes.
Panel A: Immunofluorescence staining was performed using an anti-Pax3 or anti-Meox1 antibody in order to detect the number of cells expressing these two transcription factors on day 5 (red). The nuclei were visualized by Hoechst dye (blue). Images are shown at 400x magnification and the scale bar represents 20μm. Panel B: For quantification of positive cells, 10 fields of view were counted per coverslip, in duplicates, for a total of three independent differentiations. Error bars represent the average ± SEM. Statistical analysis was performed using the Student's T-test (*versus* control cells) with a p-value of at least $p<0.05$ was considered statistically significant.

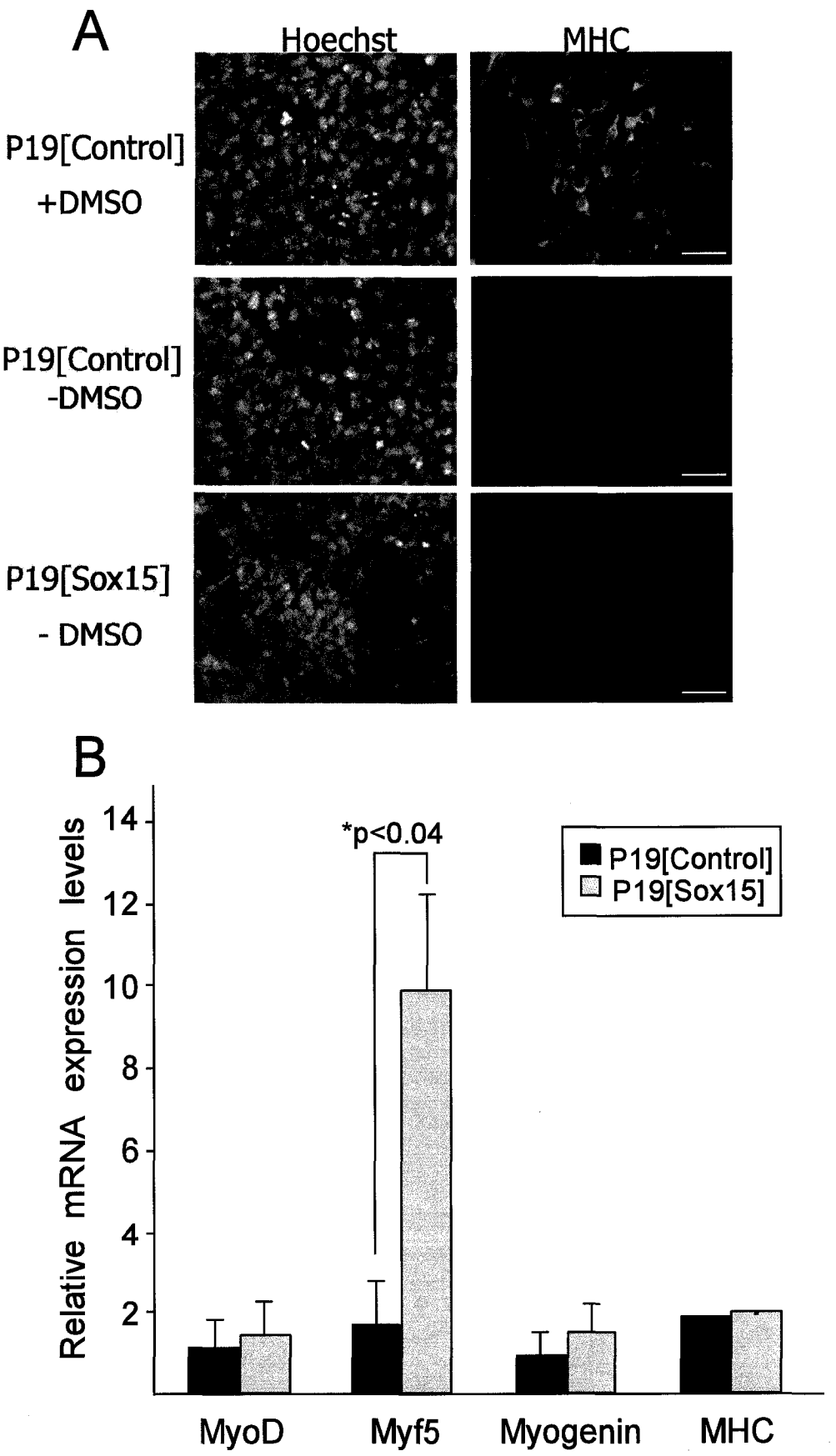


2.3.3 – Sox15 does not induce MHC expression

P19[Sox15] cells were also analyzed for their ability to undergo myogenic differentiation in the absence of DMSO, conditions which do not normally support differentiation in culture. As expected, P19[Control] cells aggregated with DMSO formed MHC-positive skeletal myocytes, while P19[Control] cells aggregated without DMSO did not form skeletal myocytes, as seen by the lack of MHC-positive staining (Figure 2.3, A). P19[Sox15] cells aggregated in the absence of DMSO and fixed on day 9 also did not differentiate into skeletal muscle, as noted by the lack of MHC staining, indicating that Sox15 could not bypass the DMSO requirement for myogenesis (Figure 2.3, A). In terms of gene regulation, MyoD, Myogenin and MHC mRNA transcripts were not induced in P19[Sox15] cultures, consistent with the phenotype of the undifferentiated P19[Control] cells (Figure 2.3, B). However, we did note a statistically significant 10-fold over background (±2, n=4) increase in Myf5 expression for the P19[Sox15] clonal populations, compared to the 2-fold (±1, n=3) seen in P19[Control] cells (Figure 2.3, B). These observations suggest that while Sox15 is able to increase the proportion of cells specified to the myogenic lineage, it is not sufficient to induce skeletal myogenesis in aggregated P19 cells.

The upregulation of Pax3/7, Meox1 and Foxc1 at early stages, but not MyoD, Myogenin or MHC at later stages suggests a positive role for Sox15 in enhancing skeletal muscle precursor formation and possibly a negative role in regulating the myoblast to myocyte differentiation program.

Figure 2.3 – Sox15 does not induce P19 cell differentiation into skeletal myocytes. P19[Control] and P19[Sox15] stable cell lines were differentiated in the absence of DMSO. Panel A: Skeletal muscle differentiation was confirmed by immunofluorescence using an antibody against MHC, and nuclei were visualized with Hoechst dye. Images are shown at 200x magnification, and the scale bar represents 20μm. Panel B: Quantitative PCR analysis of gene expression reveals a lack of MyoD, myogenin and MHC mRNA expression (data has been normalized to GAPDH and is expressed relative to P19[Control] day 0). Error bars represent *average ± SEM,* of 3 independent differentiations of 2 separate clones. *$p<0.04$, compared to P19[Control] (Student's T-test).



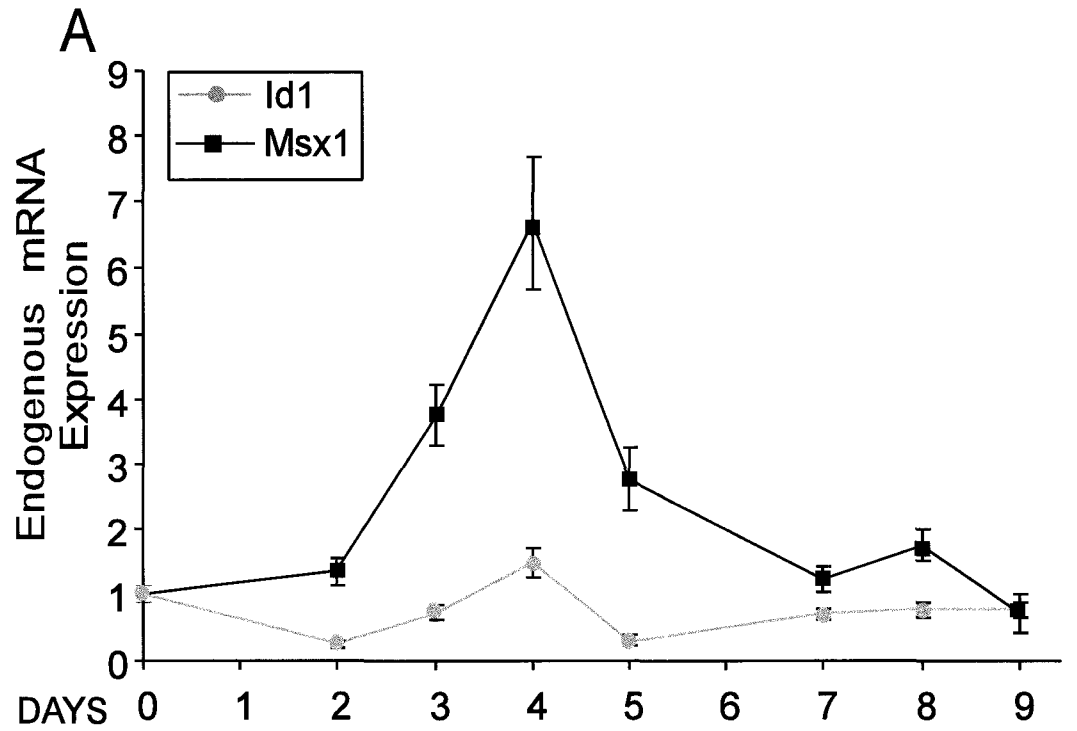
2.3.4 - Sox15 upregulates the expression of negative regulators of myogenic differentiation

In an attempt to interpret the lack of progression of P19[Sox15] cell lines past the muscle precursor stage, microarray analysis was performed to identify genes that are differentially regulated by expression of Sox15 (data not shown). Our screen detected the upregulation of Msx1 and Id1, which are known inhibitors of myogenic differentiation (3, 19, 25, 26, 43, 44). The expression levels of both Msx1 and Id1 peaked on day 4 of DMSO-induced P19 cell differentiation, and were downregulated to basal levels as differentiation progressed (Figure 2.4.1, A). However, in P19[Sox15] cell lines, both Msx1 and Id1 were elevated on day 5, compared to P19[Control] cells, after aggregation without DMSO (Figure 2.4.1, B). These levels were also substantially higher than the endogenous levels observed during EC cell differentiation (Figure 2.4.1, A). Therefore, elevated and sustained Msx1, and elevated Id1 levels on day 5 could contribute to the block observed in the myogenic progression of P19[Sox15] cells. In addition, we observed elevated and sustained levels of Wnt3a, and to a lesser extent, Wnt5a, in the presence of Sox15 (Figure 2.4.2, A and B), factors which are associated with proliferating mesodermal populations (28). Therefore, it is plausible that a combination of elevated levels of Msx1/Id1 and Wnt3a/Wnt5a contribute to the failure of P19[Sox15] cells to progress along the myogenic pathway.

2.3.5 - Myogenesis is disrupted in the presence of a dominant-negative Sox15 mutant

Loss-of-function experiments were performed by using four clonal P19[Sox15/EnR] cell lines and two P19[Control] cell lines. P19[Sox15/EnR] cell lines overexpressed a dominant-negative Sox15 fusion protein, in which the activation domain of Sox15 was

Figure 2.4.1 – Sox15 upregulates the expression of genes known to inhibit myogenesis. RNA was harvested on days 0-9 from P19 cells differentiated in the presence of DMSO, and analyzed for the expression of Id1/Msx1 by Q-PCR. Error bars represent *average ± SEM,* for reactions performed in triplicate (Panel A). P19[Sox15] and P19[Control] cells were differentiated in the absence of DMSO, and RNA isolated on days 0, 5 and 9 for gene expression analysis by Q-PCR. Error bars represent *average ± SEM,* of 3 independent differentiations of 2 separate clones (Panel B). For all of the experiments described above, the data was normalized using the internal control GAPDH, and is expressed relative to day 0.



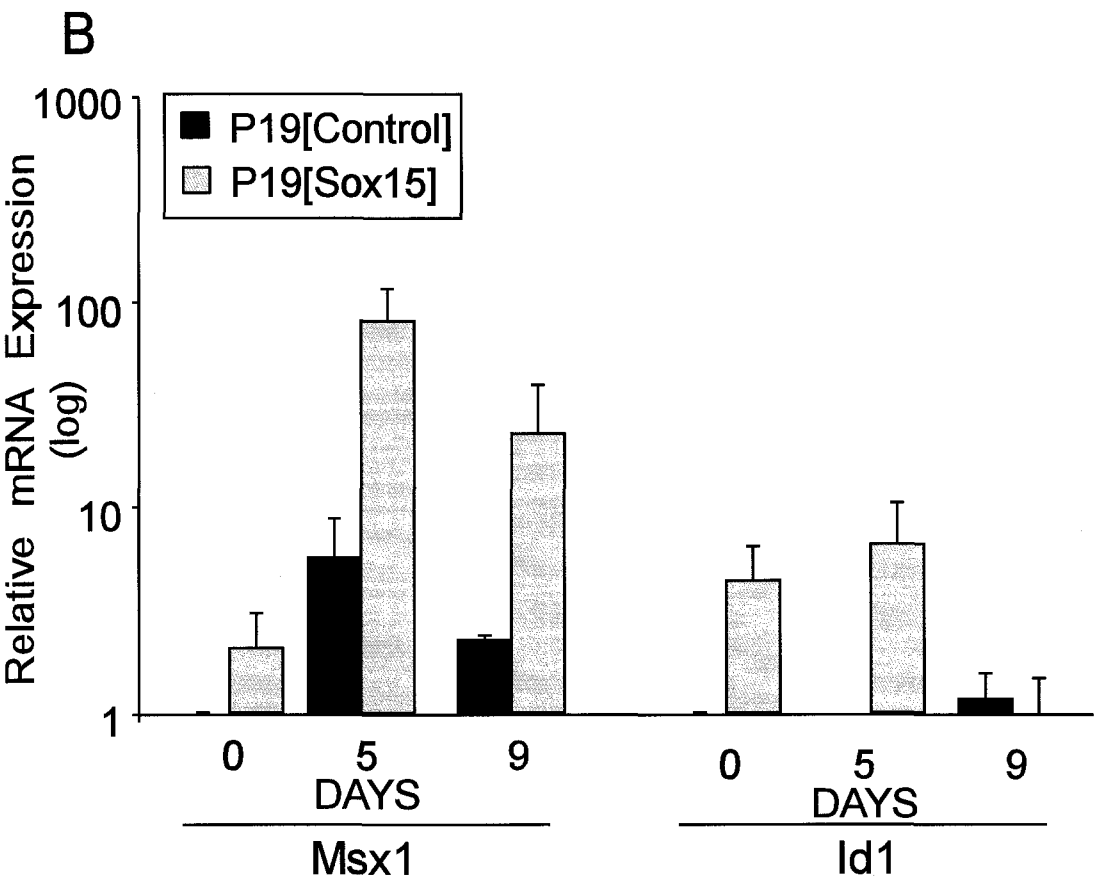
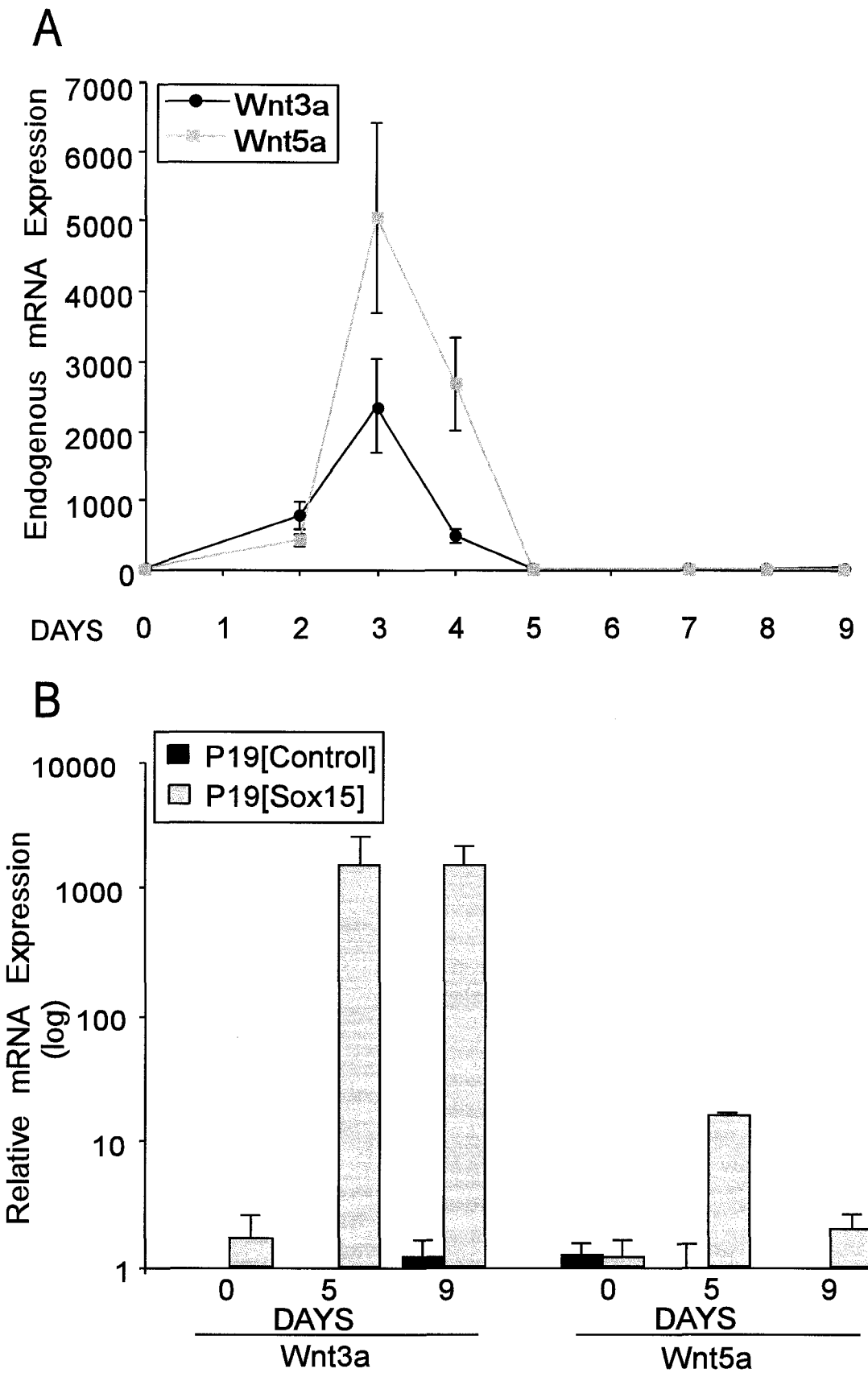


Figure 2.4.2 – Sox15 upregulates the expression of genes known to maintain proliferation. RNA was harvested on days 0-9 from P19 cells differentiated in the presence of DMSO, and analyzed for the expression of Wnt3a/Wnt5a. Error bars represent the *average ± SEM,* for reactions performed in triplicate (Panel A). P19[Sox15] and P19[Control] cells were differentiated in the absence of DMSO, and RNA isolated on days 0, 5 and 9 for gene expression analysis by Q-PCR. Error bars represent *average ± SEM,* of 3 independent differentiations of 2 separate clones (Panel B). For all of the experiments described above, the data was normalized using to GAPDH, and is expressed relative to day 0.

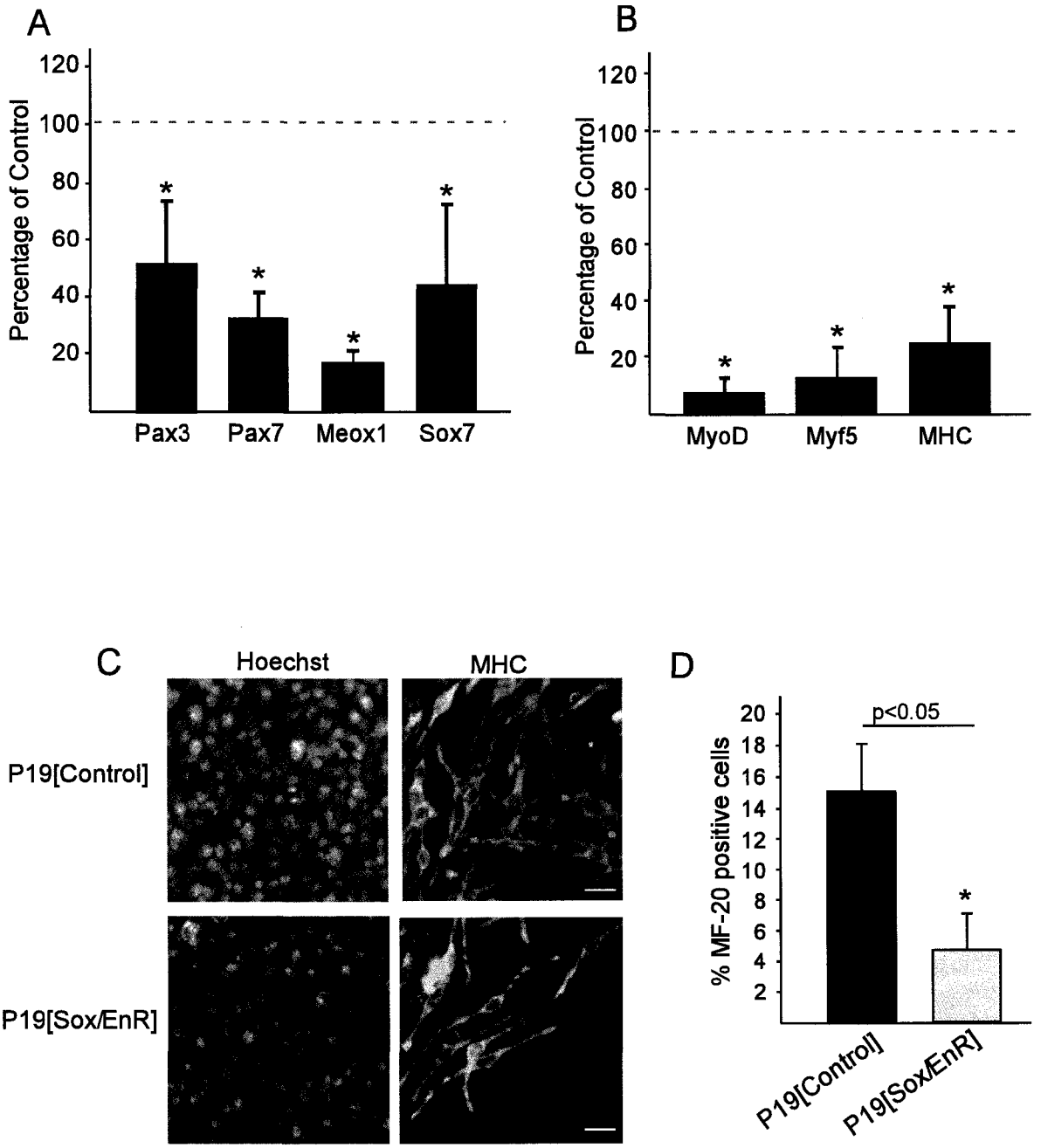


replaced with the repressor domain of the mouse EN-2 protein, as described previously (29, 30, 36). When aggregated in the presence of DMSO, Sox15/EnR clones showed an 84 ± 5% (n=4) reduction in the levels of Meox1 transcripts on day 5 of differentiation when compared to P19[Control] cultures. Further, there were statistically significant decreases in the expression of Pax3, Pax7 and Sox7 (49% ± 23%; 68% ± 9%; 56% ± 29%, n=4), respectively, in P19[Sox15/EnR] cell lines, compared to P19[Control] cells (Figure 2.5, A). In addition, myoblast formation and terminal differentiation were impaired, as demonstrated by the loss of MyoD (92% ± 5% vs P19[Control], n=3), Myf5 (87% ± 11% vs P19[Control], n=3) and MHC (75% ± 13% vs P19[Control], n=3) expression in the presence of the dominant negative mutant (Figure 2.5, B). The decrease observed in MHC mRNA expression translated into a loss of MHC protein, and an overall decrease in the number of skeletal myocytes observed in P19[Sox15/EnR] (Figure 2.5, C). Quantification of the number of MHC-positive cells revealed a 3-fold decrease in overall myogenesis in the presence of a dominant-negative Sox15 transcription factor (Figure 2.5, D). These results suggest that Sox15-regulated genes are required for proper myogenesis to occur in P19 cells.

2.3.6 - Loss of endogenous Sox15 expression does not affect overall myogenesis

To assess the role of endogenous Sox15 during myogenesis, we silenced the expression of Sox15 using RNA interference. Cells lines were generated that stably express a short-hairpin construct targeting one of two different regions of the Sox15 open reading frame (shSox15), or a scrambled sequence with no homology to any sequences in the mouse genome (shScrambled). Individual clones were picked and expanded for further analysis, or pooled together and analyzed. For the purpose of these studies, one pooled experiment was

Figure 2.5 – A dominant negative Sox15 mutant decreases overall myogenesis.
P19[Control] and P19[Sox15/EnR] cell lines were differentiated in the presence of DMSO and RNA was harvested for quantitative PCR analysis. RNA was isolated on day 5 for the detection of Pax3/7, Meox1, Foxc1, Sox7 (A) and on day 9 for MRF and MHC expression (B). The data has been normalized to GAPDH, and is reported as the percentage of P19[Control] expression. Statistical analysis versus P19[control] was carried out using the Student's T-test (*$p<0.05$). We detected the presence of myocytes in these cell lines by immunofluorescence using an antibody against MHC. Scale bars represent 20μm (C). We calculated the percentage of cells that are MHC-positive by counting 10 fields of view per coverslip from 2 independent differentiations (D). Error bars represent average ± SEM and *$p<0.05$ was determined using the Student's T-test.

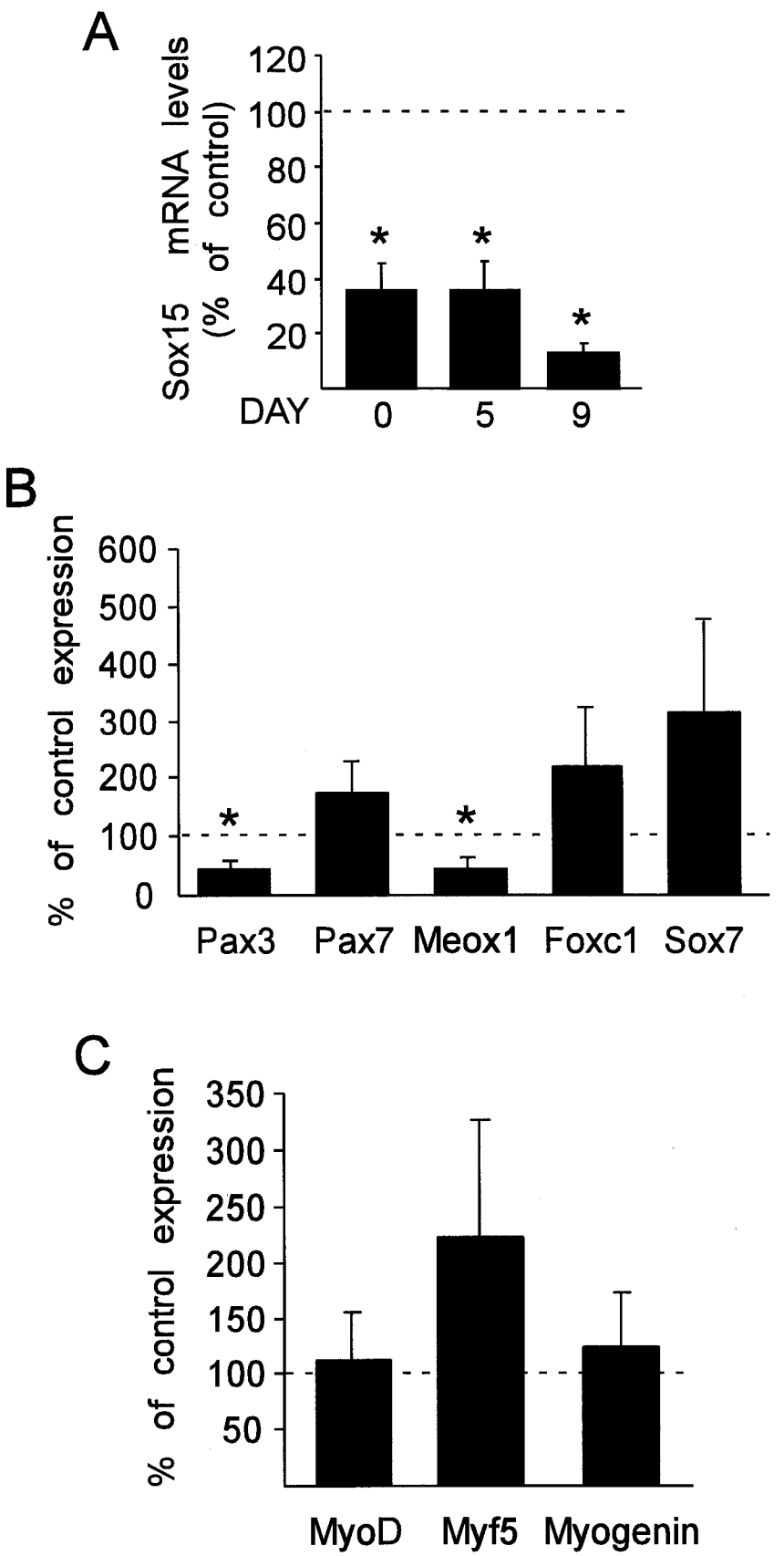


considered as one clone. Analysis of 12 clones indicated a 64% (±10%) to 87% (±3%) knockdown of Sox15 expression, over the time course of differentiation, compared to P19[shScrambled] cell lines (Figure 2.6, A). Analysis of gene expression in P19[shSox15] cell lines revealed a 46% (± 8%, n=12) decrease in Pax3 and a 47% (± 14%, n=12) decrease in Meox1 mRNA expression, when compared to P19[shScrambled] cells (Figure 2.6, B). Surprisingly, Pax7 and Foxc1 were unaffected by the knockdown of Sox15, while Sox7 appeared slightly upregulated (Figure 2.6, B). Moreover, MRF expression remained unaltered, and even appeared slightly increased in the P19[shSox15] cells, as compared to P19[shScrambled] cells (Figure 2.6, C). From these observations, we can conclude that while Sox15 appears to be required for efficient Pax3 and Meox1 expression, it is dispensable for Pax7 and Foxc1 expression. It is possible that other factors can compensate for Sox15 and regulate downstream gene expression in its absence. The fact that Sox7 expression was slightly upregulated by the decrease in Sox15, suggests that Sox15 may be a negative regulator of Sox7.

2.3.7 - Sox7 induces myogenesis in aggregated P19 cells

Given our previous observations that Sox7 is expressed during the precursor stage of myogenic differentiation in P19 cells (Figure 2.1), and that Sox7 is downregulated by Sox15/En (Figure 2.5, A) and slightly upregulated by Sox15 knockdown (Figure 2.6, B), we were interested in determining whether Sox7 could also modulate muscle differentiation. Cell lines that stably expressed Sox7, termed P19[Sox7], were generated and differentiated in the absence of DMSO. Q-PCR was used to measure the changes in gene expression that

Figure 2.6 – Interference with endogenous Sox15 expression causes a decrease in Pax3 and Meox1 gene expression. Stable cell lines expressing shRNA specific to Sox15 were differentiated in the presence of DMSO and RNA was harvested on days 0, 5, and 9. Quantitative PCR was performed to determine the efficiency of Sox15 knockdown throughout the differentiation (A) and for the expression of the indicated genes on day 5 (B) and day 9 (C) of differentiation. The error bars represent average ± SEM, n=12 clones. The data was normalized using GAPDH as in internal control and expressed as a percentage of the expression in control cell lines, for each respective day. Statistical analysis was performed using the Student's T-test (compared to the control group), with a p-value of at least $p<0.05$ considered statistically significant.

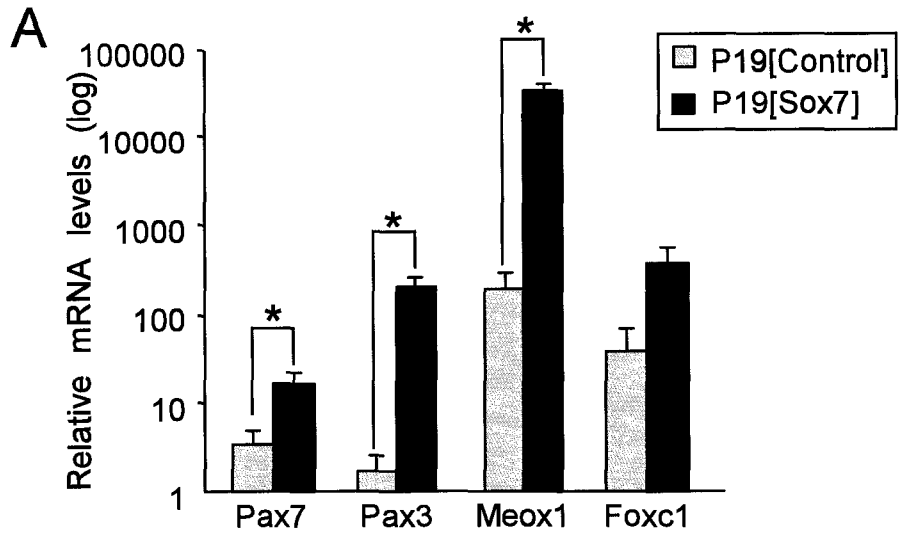


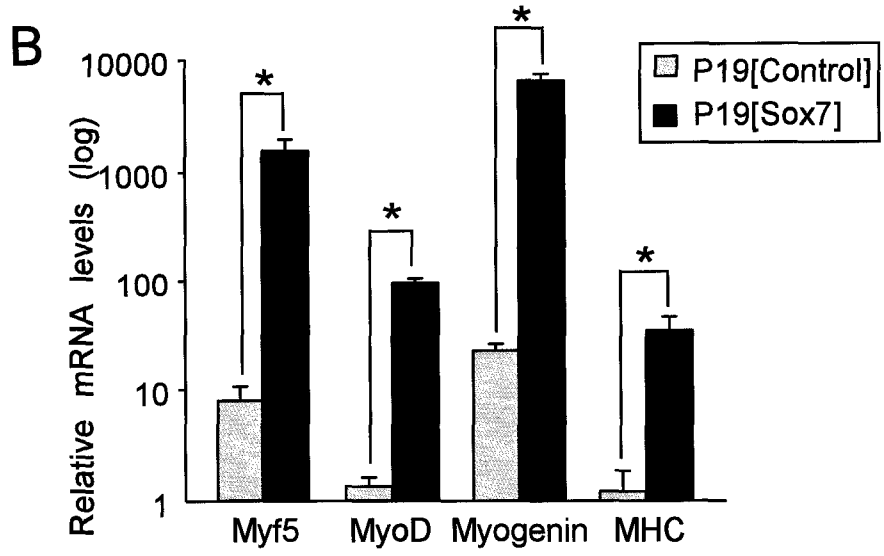
were induced by the presence of Sox7. Sox7 was overexpressed an average of 162-fold (±73, n=8) to 323-fold (±184, n=8) over the time course of differentiation (Figure 2.8, B). A significant increase in the expression of Pax3/7, Meox1 and Foxc1, markers associated with muscle precursors, were observed in aggregated P19[Sox7] cells, compared to control cells (Figure 2.7, A). Unlike the phenotype observed for P19[Sox15] cells, Sox7 was able to enhance the expression of Myf5, MyoD and Myogenin mRNA as well as enhance MHC transcript expression (Figure 2.7, B). Using an anti-MHC antibody, we were also able to detect the presence of fully differentiated myocytes in P19[Sox7] cells, as compared to P19[Control] cells in the absence of DMSO (Figure 2.7, C). Quantification of the number of MHC-positive cells in the total population revealed that in the presence of Sox7, 5% (± 1%, n=4) of the total cell population differentiated into MHC-positive skeletal myocytes as compared to P19[Control] cells which did not form muscle (Figure 2.7, D). Therefore, Sox7 was able to induce skeletal myogenesis in aggregated P19 cells and upregulate the expression of Pax3/7, Meox1, MRFs, and MHC.

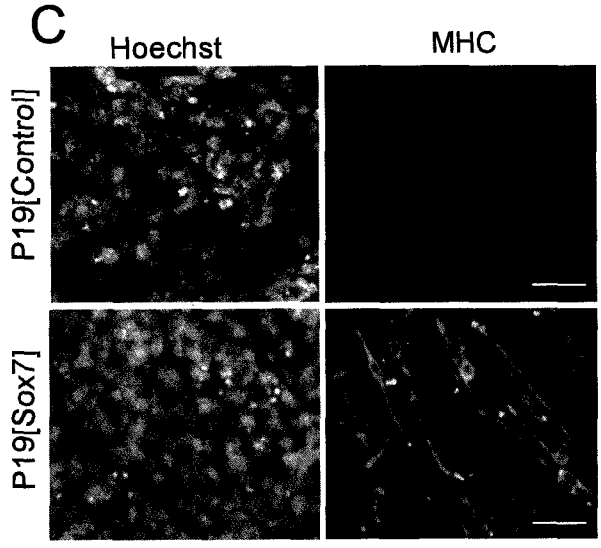
2.3.8 - Sox15 binds the genomic region 5' to the transcriptional start site of Sox7

Given that Sox7 expression is downregulated in the presence of a dominant-negative Sox15 mutant, and slightly upregulated with the knock down of Sox15, we further investigated the possibility that Sox7 expression is controlled by Sox15. Using Q-PCR, we measured the levels of Sox7 transcripts in P19[Sox15] cells, and the amount of Sox15 transcripts in P19[Sox7] cells. Expression of Sox15 in P19[Sox7] cells was significantly decreased compared to P19[Control] cells, which is consistent with our observations that endogenous Sox15 levels were decreased during the differentiation process (Figure 2.8, A).

Figure 2.7 – Sox7 upregulates genes found in preskeletal mesoderm, myoblasts, and myocytes, resulting in the induction of skeletal myogenesis. P19[Control] and P19[Sox7] cell lines were differentiated in the absence of DMSO. Panels A & B: Quantitative PCR analysis of mRNA levels was performed on day 5 for the detection of Pax3/7, Meox1 and Foxc1, and on day 9 for the detection of the MRFs and MHC. The data was analyzed by normalizing to GAPDH and expressing the results relative to day 0 of P19[Control] cells. The error bars represent the *average ± SEM,* for 2 P19[Control] and 3 P19[Sox7] clones from 2 independent differentiations. Statistical analysis was performed using the Student's T-test, with a p-value of at least $p<0.05$ considered statistically significant. Panel C: Immunofluorescence was performed by fixing cells on day 9 of differentiation and staining the coverslips using an anti-MHC antibody. Scale bar represents 20μm. Panel D: Ten fields of view per coverslip (in duplicate) were counted for 4 P19[Sox7] cell lines and 3 P19[Control] cell lines. Statistical significance of $p<0.02$ was calculated using the Student's T-test.







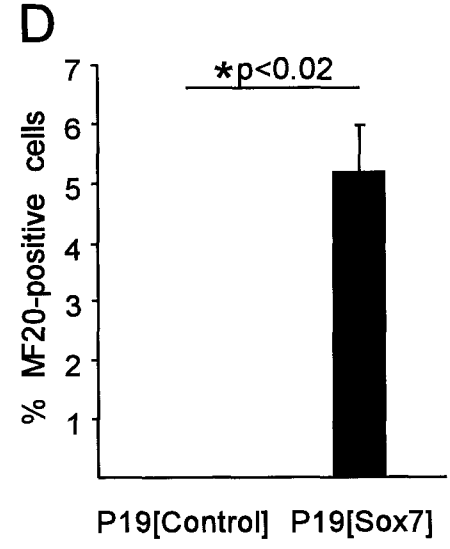
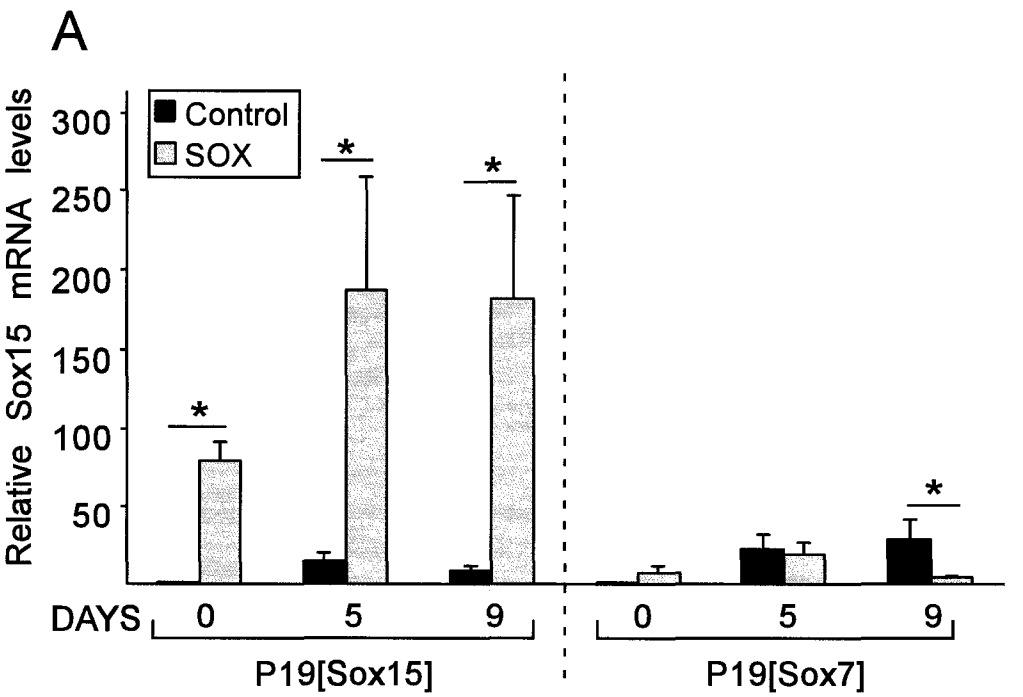
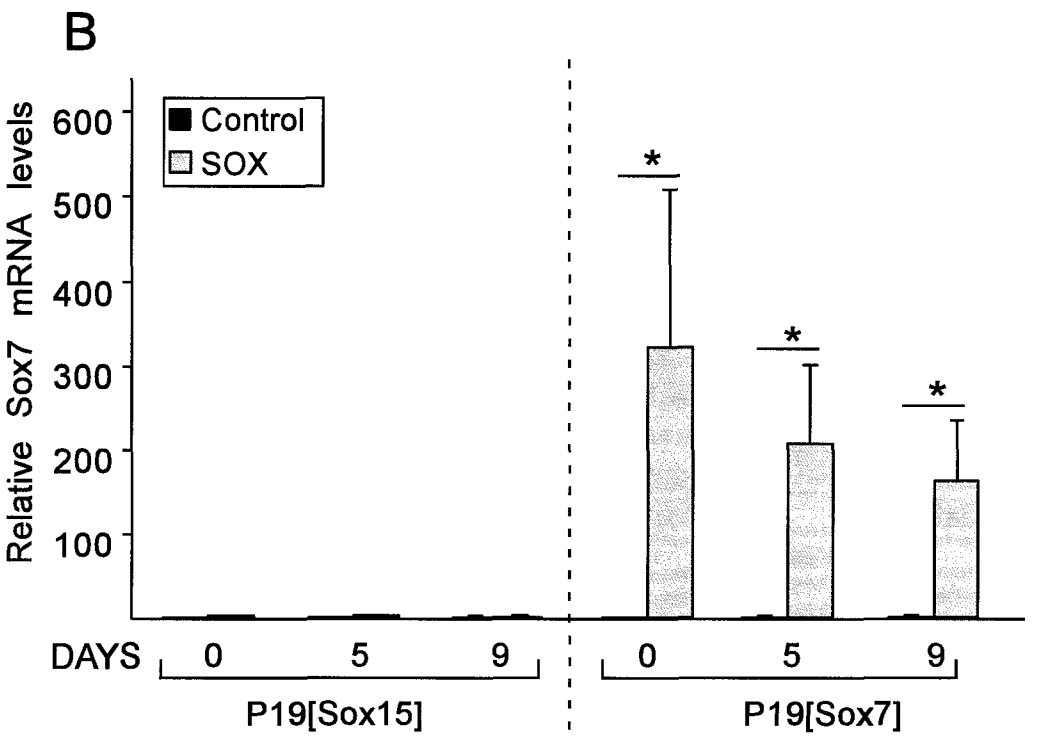


Figure 2.8 – Sox15 does not induce the expression of Sox7. Using quantitative PCR, we measured the relative mRNA levels of Sox15 in P19[Sox7] cells (A) and Sox7 in P19[Sox15] cell lines (B) differentiated in the absence of DMSO. Error bars represent average ± SEM. Statistical analysis versus control at each time point was carried out using the Student's T-test.





While Sox15 was sufficient to induce Pax3/7, Meox1 and Foxc1 transcript levels from 10 to 500-fold over background levels (Fig. 2.2.1), Sox15 only enhanced Sox7 expression levels 2-fold (±1, n=5), compared to the 0.3-fold (±0.1, n=3) fold change in P19[Control] cells (Figure 2.8, B). The 2-fold induction is in marked contrast to the 36-fold (±11) enhancement observed on day 5 of DMSO-induced P19 cell differentiation (Figure 2.1, A). Thus Sox15 does not upregulate Sox7 strongly compared to other transcription factors expressed at a similar stage of skeletal myogenesis.

To characterize the role of Sox15 in regulating Sox7 expression, we used ChIP to determine if Sox15 occupies the chromatin upstream of the mouse Sox7 gene. Using MULAN, we identified one conserved Sox binding site located within a conserved element approximately 11kb upstream of the Sox7 transcriptional start site. Using an antibody against Sox15, a 3-fold ±1 (n=5) enrichment in chromatin fragments corresponding to the Sox site located at -10,857 bp 5' of Sox7 was observed compared to immunoprecipitations performed using a goat IgG as a negative control (Figure 2.9). Additionally, *in silico* studies revealed the presence of a conserved Sox binding site upstream of the Pax3 start site, which did not associate with Sox15, as determined by ChIP. Thus, Sox15 appears to bind to a conserved element in the Sox7 gene, but not the Pax3 gene, in a population of differentiating P19 cells.

2.4 - Discussion

We have shown that Sox15 was sufficient to upregulate the expression of markers of skeletal muscle precursors in P19 cells under non-muscle inducing conditions. However, these cells, which have elevated/sustained levels of the myogenic inhibitors Id1 and Msx1,

Figure 2.9 – Sox15 binds a conserved region of the Sox7 gene during differentiation. *In silico* studies using MULAN identified one evolutionarily conserved Sox binding site upstream of the Sox7 transcriptional start site. Chromatin immunoprecipitation was performed using an anti-Sox15 antibody to identify enriched Sox15 targets, compared to mock IP samples, in P19[Sox15] cells on day 5 of differentiation. Sox15 appears to bind to conserved regions in the Sox7 gene but not to conserved regions in the Pax3 gene. *$p<0.05$, Student's T-test.

```
                                5'                                                3'
H. sapiens   (-9058)    TGATCTTTGTGCTATTGTTGTCATAAAATTTTACTT
M. musculus  (-10870)   TGGTCTTTGTGCTATTGTTATTACACCATTTTACTT
                        ** ***************** * * *  *********
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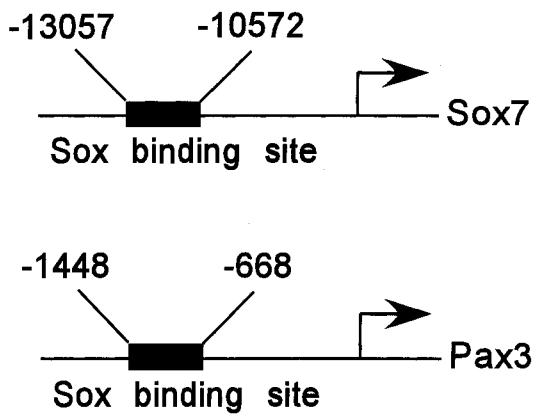

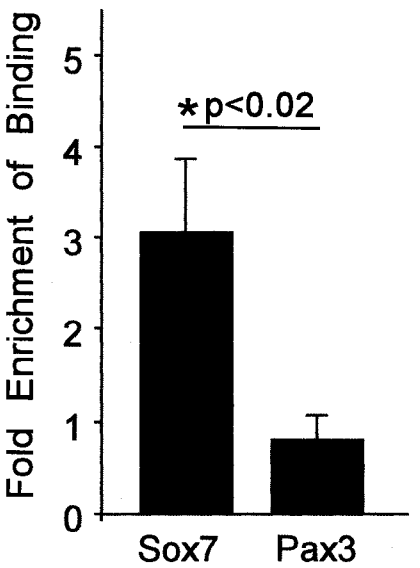

failed to progress past the myoblast stage and did not express MyoD, myogenin or MHC. In the presence of a dominant-negative Sox15, a reduction in Meox1, Pax3/7 and Foxc1 transcripts was observed as well as a decrease in MRF expression and overall myogenesis, as noted by the decline in the number of MHC-positive cells. The knock down of endogenous Sox15 expression on the other hand, resulted in a decrease of Pax3 and Meox1 only, suggesting that other factors may compensate for the lack of Sox15 and regulate muscle precursor cell fate and subsequent differentiation. Ectopic expression of Sox7 was sufficient to initiate and direct the entire myogenic program, giving rise to MHC-positive skeletal myocytes under non-muscle inducing conditions.

These findings are consistent with a model in which Sox15 may regulate the expression of Pax3/7, Meox1 and Foxc1, and therefore may function positively to enhance the specification of cells into the myogenic lineage. In agreement with previous results (4), we observed that cells expressing sustained, high levels of Sox15 failed to progress past the precursor/myoblast stage. Further, P19[Sox15] cell lines did not enhance Sox7 expression levels, and knockdown of Sox15 slightly upregulated Sox7. It is possible that Sox15 is acting as a repressor of Sox7 expression under these circumstances. This model agrees with our observations that cell lines expressing high levels of Sox15 and low levels of Sox7 fail to terminally differentiate, whereas cell lines expressing low levels of Sox15 and high levels of Sox7 exhibit normal myogenesis.

Our finding that Sox15 can regulate Pax3 expression and thus enhance specification into the myogenic lineage is consistent with the phenotype of mice lacking Sox15. These mice show a deficiency in MyoD but not Myf-5 expression in primary myoblasts, implying a lack of specification of MyoD-expressing myoblasts (20). Interestingly, embryonic expression of Meox1 or Pax3 was not examined in $Sox15^{-/-}$ mice (20). We would predict a downregulation

of Pax3 and Meox1 expression in the developing DM, although compensation by Sox7,8, 9, or 11 may confound the outcome (4, 20, 37)

Gene expression analysis revealed that Wnt3a expression is elevated and sustained in P19[Sox15] cell lines, and that these cells failed to progress through the myogenic pathway. Recent studies have demonstrated that the expression of a constitutively active β-catenin in satellite cells caused an increase in the proportion of cells expressing Pax7, without a concomitant increase in MyoD. These results suggest that β-catenin promotes self-renewal of muscle precursors, with fewer cells undergoing myogenesis (28). It is tempting to speculate that the elevated levels of Wnt3a observed in our system are contributing to the maintenance of muscle precursors at the expense of differentiation.

Our finding that Sox15 failed to induce myogenesis in P19[Sox15] cells aggregated in the absence of DMSO is consistent with the findings of Lee *et al.*, who determined that ectopic expression of Sox15 is not sufficient to initiate the myogenic program (20). They showed that stable expression of Sox15 in 3T3 fibroblasts did not result in positive MHC staining, or any detectable MyoD/Myf5 expression. In other studies, ectopic expression of Sox15 in C2C12 proliferating myoblasts antagonized differentiation and decreased MyoD expression, whereas a C-terminal truncation of Sox15 showed no adverse effects on myogenesis (4). Finally, Sox15 expression decreased during myoblast differentiation. Therefore, in these studies, increased levels of Sox15 appeared inhibitory towards myoblast maintenance and differentiation (4).

Similarly, in our experiments, Sox15 seemed to inhibit MyoD expression, since an increase in Pax3 expression did not result in a concomitant increase in MyoD expression, as would be predicted from the phenotype of P19[Pax3] cells (36). Our results are consistent

with a model in which Sox15 enhances Pax3 and Meox1 expression during muscle specification and then inhibits myoblast maintenance and/or differentiation if not subsequently downregulated. A dual positive/negative function for stem cell transcription factors is also observed during melanocyte stem cell differentiation. In this case, Pax3 acts early to activate expression of Mitf, while simultaneously inhibiting expression of melanin-synthesizing genes. These cells are thus committed, yet will not differentiate until the Pax3-mediated inhibition is relieved (18). Alternatively, the lack of upregulation of MyoD in the current study may be due to the presence of an uncharacterized inhibitor or the absence of an essential activator.

Since disruption of wild-type Sox15 function resulted in a decrease of Meox1 and Pax3 expression, it is possible that these two genes are immediate downstream targets of Sox15, or that their expression cannot be compensated by the presence of another transcription factor. Attempts to determine Pax3 and Meox1 promoter occupancy by Sox15 using chromatin immunoprecipitation (ChIP) were unsuccessful (Figure 2.9 and data not shown). In this case, Pax3/Meox1 are likely indirect targets, although regions outside of the 25 Kb promoter region were not examined. In contrast, Sox7 appears to be a direct target of Sox15 and expression analysis indicates that Sox15 may be a negative regulator of Sox7 expression. Further studies are required to elucidate the network of direct and indirect factors regulated by Sox15 and Sox7, ideally using high throughput methods.

In summary, we have provided evidence that Sox15 and Sox7 may be regulators of early muscle precursor cell fate by functioning as upstream regulators of Pax3/7, Meox1 and Foxc1 expression. This regulation may enhance the specification of cells into the muscle lineage. Furthermore, Sox7, but not Sox15, regulates myoblast formation and differentiation by upregulating MRF and MHC expression. Thus, we propose differential roles for Sox15

and Sox7 during myogenesis. Sox15 appears to initiate and maintain skeletal muscle precursor cell formation whereas Sox7 can initiate the entire myogenic program. These findings could have implications for future stem cell therapy which requires the ability to control differentiation versus proliferation during myogenesis.

2.5 Acknowledgments

We wish to thank Jennifer Dawson for critically reading the manuscript. The authors also wish to acknowledge the technical assistance of Sophie Boisvenue and Flavia Sendi-Mukasa. J.S. was supported by a NSERC studentship as well as a Canadian Institute of Health Doctoral Research Award (Institute of Aging). I.S.S. was supported by a Canadian Institute of Aging Investigator Award. This work was supported by grant MOP-49605 (to I. S. S.) from the Neuromuscular Research Partnership, an initiative of ALS Canada, Muscular Dystrophy Association of Canada, and the Canadian Institutes of Health Research.

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Chapter 3

Canonical Wnt signaling regulates Foxc1/2 expression in P19 cells[1]

[1]A version of this chapter has been submitted

Savage, J., Voronova, A., and Skerjanc, I.S., (2008) Canonical Wnt signaling regulates Foxc1/2 expression in P19 cells. *Submitted to Differentiation*

3.1 – INTRODUCTION

During embryogenesis, signals emanating from the notochord, the floor plate of the neural tube as well as the dorsal ectoderm are important for proper patterning of the paraxial mesoderm, which eventually gives rise to the somites. Cells originating from the dermomyotome, one of the somitic compartments, eventually migrate laterally to form the myotome, which comprises the first differentiated skeletal myocytes of the developing embryo. In order for these events to occur, the expression of transcription factors and signaling molecules requires precise regulation. Maturation of the somite into these specific compartments as well as myoblast formation is a result of tissue interaction and integration of positive and inhibitory stimuli such as Wnts, BMPs and Sonic Hedgehog (Shh). The molecules are secreted from the overlying ectoderm as well as the neural tube and the notochord (14, 15, 47). These extracellular signals activate a cascade of transcription factors that ultimately leads to expression of the myogenic regulatory factors (MRFs), MyoD, Myf5, myogenin and MRF4, which control the end-stages of myogenesis (3). Induction of MRF expression in response to extra-cellular cues appears to be mediated by several transcription factors including Pax3 (31), Meox1/2 (11), Six1 (28) and Gli2 (9).

One transcription factor that has emerged as being an important regulator of paraxial mesoderm cell fate and patterning belongs to the Forkhead family of genes. Foxc1 contains a highly conserved 110 amino acid DNA binding motif (50). Crystallography studies have determined that the forkhead domain adopts a "winged-helix" motif, which is essentially a helix-turn-helix core of three α-helices flanked by two loops(13). Unlike helix-loop-helix proteins, Fox family members bind DNA as monomers inducing a dramatic bend (80-90 degrees) in the DNA (37). Target DNA sequences are usually comprised of a seven

nucleotide core RYMAAYA (R=A or G, Y=C or T, M=A or C). However, this core sequence is not sufficient for high affinity binding, but also depends on flanking sequences on both sides of the core (23). Some groups of forkhead proteins have nearly identical DNA binding domains, so similar in fact that the specificities might be assumed to be the same. One example is Foxc1 and Foxc2 which share 97% amino acid homology in the forkhead domain (26). Fox transcription factors have been shown to act mostly as transcriptional activators, but some proteins have been found to act as negative regulators of gene expression, particularly FoxD2, -D3 and –G1(10, 18, 44).

Forkhead transcription factors have been implicated in the regulation of many developmental processes including foregut morphogenesis, proper lens formation, hair follicle development and cell cycle regulation (2, 5, 21, 33). In particular, Foxc1 and Foxc2 have been implicated in regulating somitic development, and show, to some extent, redundant biological roles. These two transcription factors are expressed in the paraxial mesoderm and somites (20, 45, 52). $Foxc1^{lacz}$ mice die pre- and perinataly with noticeable kidney, eye, cardiac and skeletal defects and $Foxc2^{lacz}$ mice show a similar spectrum of prenatal developmental abnormalities (25, 53). $Foxc1^{-/-}$;$Foxc2^{-/-}$ homozygous mutant mice have a more severe phenotype, depicted by the lack of expression of segmentation genes that are normally transcribed in anterior and posterior regions of the somites. In this case, the somites fail to form and expression of the myogenic marker MyoD is lost in the compound homozygous animals, although expression of mesodermal genes, such as Meox1, is maintained (27). Deletion of the zebrafish homolog *foxc1a* also affects the anterior-posterior patterning of the presumptive somites and disrupts boundary formation (48). Conversely, forced expression of Foxc1 in chick intermediate mesoderm is sufficient to change cell fate towards paraxial mesoderm, upregulating transcription factors such as Paraxis and Pax7 (51).

Taken together, these studies suggest that the expression of Foxc1 and Foxc2 is crucial for somitogenesis in the developing embryo.

Given the important role of Foxc1/2 in regulating somitogenesis, we sought to determine the upstream mechanisms responsible for regulating the expression of these two genes. By placing Foxc1/2 within the cascade of transcription factors that regulate somite development and skeletal myogenesis, we will gain a better understanding of its physiological role during development. Here β-catenin was found to be essential for the expression of Foxc1/2 during myogenesis in P19 cells, while Gli2 and Meox1 are involved in the maintenance of Foxc1 transcription.

3.2 – Materials and Methods

3.2.1 – Cell Culture

P19 Embryonal Carcinoma cells (ATCC: #CRL-1825) were cultured in alpha-essential media which was supplemented with a combination of 5% fetal calf serum (PAA Laboratories, Etobicoke, Canada) and 5% cosmic calf serum (Hyclone, Logan, UT). The P19 cell lines used in this study stably overexpress dominant-negative transcription factors. The dominant-negative transcription factors are chimeric proteins where the activation domain of either Gli2, Meox1 or β-catenin, has been removed and replaced by the engrailed repressor domain. P19[Control], P19[Gli2], P19[Gli/EnR], P19[Meox/EnR], P19[β-catenin/EnR] cell lines used in this study have previously been described (35, 36, 41). For the differentiation process, cells were aggregated in the presence of 0.8% DMSO for 9 days as previously described (35).

3.2.2 – Northern blot analysis

RNA was harvested daily during the differentiation period from day 0 to day 9 using the LiCl/Urea method. A total of 12 μg of RNA for each sample was separated by denaturing gel electrophoresis using formaldehyde and, by capillary action, transferred to Hybond-N nylon membranes (GE Life Sciences, Baie d'Urfé, Québec) and cross-linked using UV irradiation. For Northern blot analysis, the cDNA fragments were labeled by incorporating α-^{32}P dCTP radioisotopes using the Megaprime DNA labeling kit (GE Life Sciences, Baie d'Urfé, Québec). G-50 microspin columns (GE Life Sciences, Baie d'Urfé, Québec) were used to remove any unincorporated nucleotides. The membranes were hybridized with the labeled cDNAs overnight at 42°C, and unhybridized cDNA fragments were removed by washing with a 2X SSC, 0.2% SDS solution at 42°C, followed by washing with 0.1X SSC, 0.2% SDS at 65°C. The membranes were exposed to autoradiography film (GE Life Sciences, Baie d'Urfé, Québec) and quantitative analyses were done using NIH Image 1.63 software (http://rsb.info.nih.gov/nih-image). Probes for Meox1, Gli2, β-catenin/EnR, engrailed, MyoD, myogenin, Pax3 and 18S have been previously described (41). The Foxc1 probe is an 1877 bp *Pst1* fragment of the mouse Foxc1 cDNA. Results shown are representative of 3 separate clones for each cell line.

3.2.3 – Reverse Transcription and Quantitative PCR

Total RNA was further purified using the RNeasy mini kit (Qiagen, Mississauga, ON) and 1μg was used for the first strand synthesis reaction. The cDNAs were generated using the Quantitect Reverse Transcription kit (Qiagen, Mississauga, ON), which includes a DNAse I treatment step. For the real-time PCR reactions, 1/20th of the cDNA mixture was

used as a template for amplification with FastStart SYBR Green with ROX (Roche Applied Sciences, Laval, Québec). Please refer to the Appendix for primer sequences.

All reactions and data analysis were performed on the ABI 7300 system (Applied Biosystems, Streetsville, ON, Canada) using SDS software. Relative gene expression was calculated using the comparative Ct method as previously described (29). All reactions were performed in duplicate, and the results shown are the average +/- SEM of three independent experiments.

3.2.4 – Immunofluorescence

On day four of the differentiation process, cells were transferred to gelatin-coated coverslips and subsequently fixed on day 9 using -20°C methanol. Immunofluorescence was performed by incubating cells with a mouse monoclonal anti-Myosin Heavy Chain antibody (MF20) at room temperature for 1 hour. After extensive washing with PBS, the coverslips were incubated in a 1:100 dilution of goat anti-mouse Cy3-linked secondary antibody in PBS (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature. The coverslips were washed, mounted and fluorescence detected using a Zeiss Axioscope microscope. Images were captured on a Sony 3CCD camera and processed with Axiovision, Adobe Photoshop7 and Canvas 8.

3.2.5 – Chromatin Immunoprecipitation

To prepare the chromatin, ten 150 mm dishes of day 2 aggregates were fixed according to the protocol in section 2.2.7. For immunoprecipitation, 5 μg of β-catenin antibody (clone 15B8, Sigma-Aldrich) or 5 μg of IgG antiserum (Zymed Laboratories, California), as a

negative control, was used. Enrichment of binding sites was analyzed using SYBR Green real-time PCR, as described above. Please refer to the Appendix for a list of primers used in this experiment.

3.2.6 Statistical Analysis

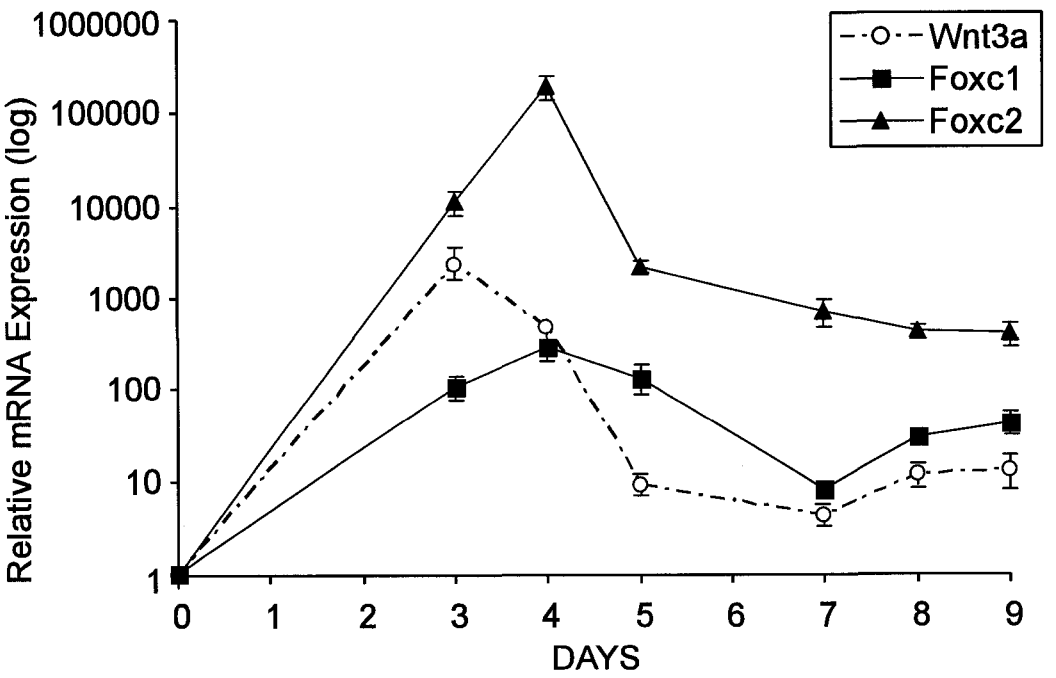
Where indicated, we determined statistical significance of the data using a Student's T-Test to compare the means from two different groups.

3.3 RESULTS

3.3.1 Foxc1 and Foxc2 expression overlap temporally with Wnt3a expression

In order to study the regulation of Foxc1/2 transcription, we first needed to establish the temporal pattern of expression. To address this issue, P19 cells were differentiated in the presence of DMSO, and gene expression was analyzed by quantitative PCR. Foxc1 mRNA transcripts were detected as early as day 3 of differentiation, with peak levels of expression on day 4 (Figure 3.1). Foxc2 is expressed in a pattern similar to Foxc1, also peaking in expression on day 4. In order to compare Foxc1/2 expression to the known pattern of mesoderm factor expression in P19 cell differentiation, the Foxc1/2 expression patterns were compared to that of Wnt3a. Interestingly, the onset of Foxc1/2 expression coincides with the expression of Wnt3a transcripts (Figure 3.1). Early expression of Foxc1 and Foxc2 in our system is consistent with its role in mesoderm specification (20, 27, 51, 52).

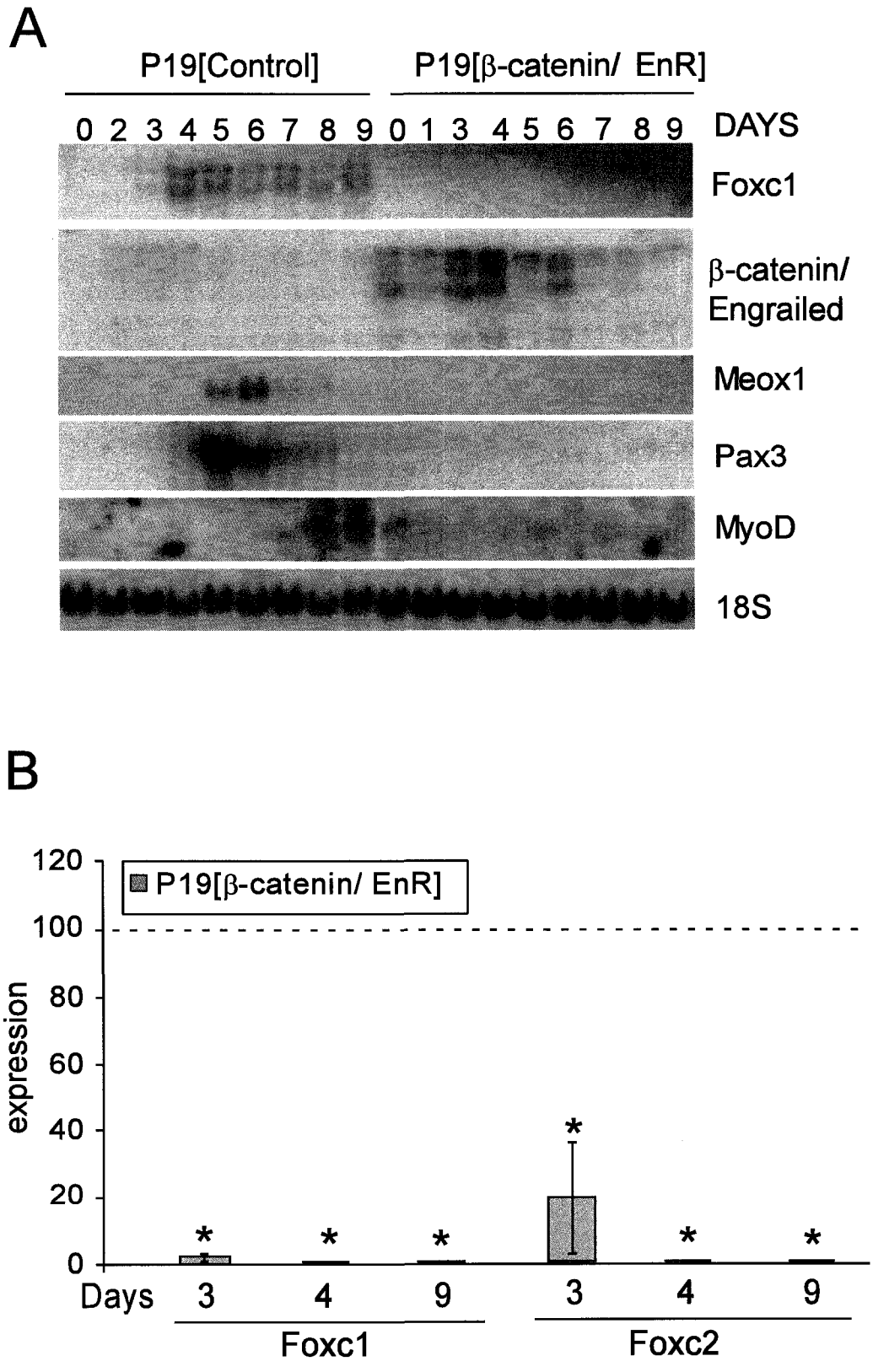
Figure 3.1 – Foxc1 and Foxc2 are expressed dynamically during P19 skeletal muscle differentiation. RNA isolated from P19 cells differentiated in the presence of DMSO was analyzed for the expression of Foxc1, Foxc2 and Wnt3a by real-time PCR. Results are normalized to GAPDH and expressed relative to Day 0. All three genes are expressed early during the time course and in an overlapping temporal pattern. Error bars represent *average ± SEM.* Reactions for this experiment were performed in triplicate.



3.3.2 β-catenin regulates Foxc1/2 expression

Since we have established that Foxc1 and Foxc2 are expressed at the same time as Wnt3a, we decided to further examine the role of the canonical Wnt signaling pathway in regulating Foxc1/2 gene expression. P19[β-catenin/EnR] cell lines overexpress a dominant-negative chimeric protein in which the β-catenin activation domain has been fused to the engrailed repressor domain. When differentiated under muscle-inducing conditions, myogenesis is abolished in P19[β-catenin/EnR] along with Pax3, Meox1 and Gli2 expression (36). To determine if β-catenin signaling can affect Foxc1/2 expression, RNA was harvested from days 0 to 9 of differentiation for Northern blot and real-time PCR analysis. As shown previously, the inhibition of β-catenin function results in a loss of Pax3, Meox1 and MyoD mRNA expression (Figure 3.2.1, A). Using real-time PCR to measure Foxc1 and Foxc2 transcript levels, we observed a 98% (± 1%, n=3) decrease in Foxc1 expression on day 3 in the presence of a dominant-negative β-catenin, when compared to P19[control] cells (Figure 3.2.1, B), and over time, Foxc1 transcripts are undetectable by day 4. Similar trends in temporal expression were observed for Foxc2 transcripts in the P19[ß-catenin/EnR] cell lines. In this case, expression is also completely abolished by day 4 of differentiation (Figure 3.2.1, B). The absence of Foxc1/2 transcripts in the presence of a dominant-negative β-catenin transcription factor suggests that Foxc1 may lie downstream of the canonical Wnt signaling pathway. To test this hypothesis, P19 cells were aggregated and either received no treatment, or were treated with 20mM LiCl, a GSK3 inhibitor previously shown to activate Wnt signaling (24). Treatment with LiCl was sufficient to induce a 36-fold (± 11, n=3) increase in Foxc1 by day 3 of differentiation. Foxc1 transcripts continued to accumulate

Figure 3.2.1 – A dominant-negative β-catenin mutant inhibits skeletal myogenesis. P19[Control] and P19[β-catenin/EnR] cell lines were aggregated in the presence of DMSO. 12μg of RNA were hybridized with labeled cDNA fragments corresponding to the factors indicated on the right. Results shown are representative of three different P19[ß-catenin/EnR] clonal populations (A). The decrease in Foxc1 and Foxc2 mRNA levels was measured using real-time PCR, normalized to GAPDH and expressed as a percentage of control cells on the same day of differentiation. Results shown are the average of three separate cell lines +/- SEM. Statistical analysis versus control at each time point was carried out using Student's T-test, *$p<0.02$ (B).

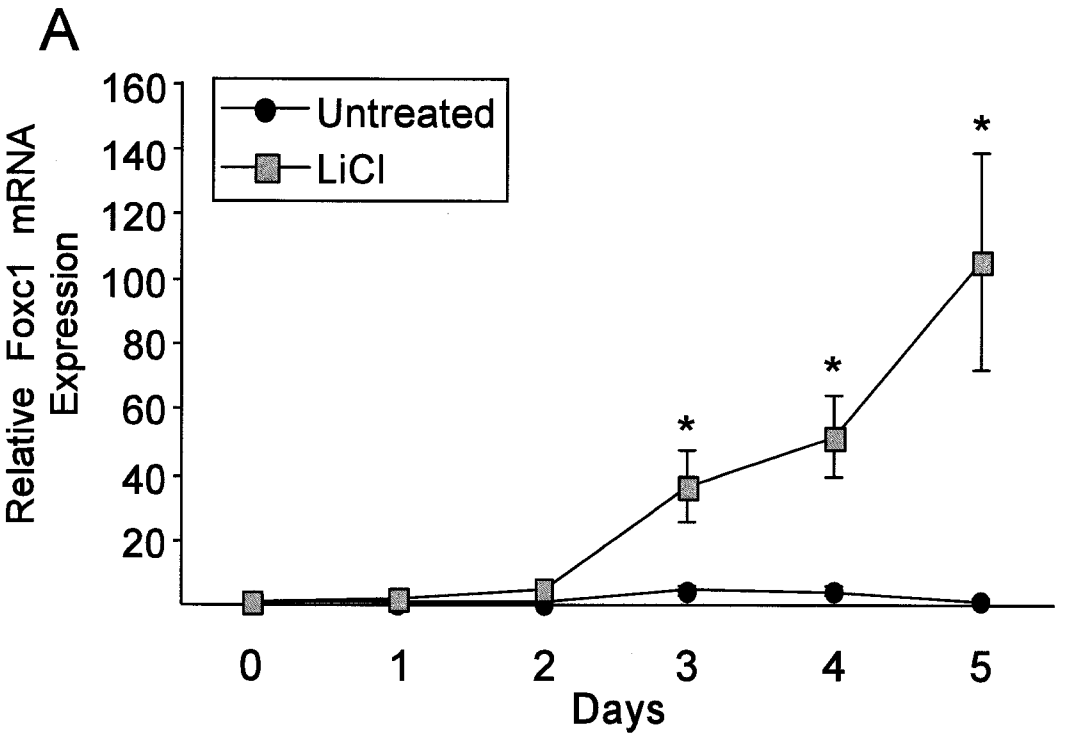


over time, peaking at 105-fold (± 33, n=3) on day 5 (Figure 3.2.2, A). Foxc2 was also induced on day 3 (20-fold, ±10, n=3) and expression continued to increase during differentiation (Figure 3.2.2, B), peaking at 201-fold (± 40, n=3). Taken together, the information obtained from the loss-of-function and gain-of-function experiments suggest that Foxc1 and Foxc2 may be targets of the canonical Wnt signaling pathway, either direct or indirect.

3.3.3 β-catenin is present at conserved LEF/TCF binding sites upstream of Foxc1

To identify conserved LEF/TCF binding sites upstream of the Foxc1 transcriptional start site, DNA sequences from the mouse and human genomes were aligned using MuLAN (http://mulan.dcode.org/), and conserved transcription factor binding sites were identified using the multiTF application (Figure 3.3, A). To determine whether canonical Wnt signaling regulates endogenous Foxc1 expression, we used Chromatin Immunoprecipitation (ChIP) to examine chromatin occupancy at conserved genomic sequences upstream of Foxc1. Using an antibody against β-catenin, we determined that β-catenin is bound to the most proximal LEF/TCF binding site (site A) in the Foxc1 promoter on day 2 of differentiation (Figure 3.3, B), while no significant binding was detected in undifferentiated P19 cells. Approximately 0.05% (±0.02%, n=3) of input sites were occupied by β-catenin, which is significantly over the background binding observed at the non-related GAPDH locus (0.01% ± 0.006%, n=3). Therefore, it appears that the canonical Wnt pathway regulates the expression of Foxc1 by a direct interaction of β-catenin with the Foxc1 promoter during skeletal myogenesis in P19 cells.

Figure 3.2.2 – Activation of the canonical Wnt signaling pathway induces the expression of Foxc1 and Foxc2. RNA from aggregated P19 cells (untreated or treated with 20 mM LiCl) was quantified using real-time PCR. Results were normalized to GAPDH and expressed relative to day 0 samples with the same treatment (n=3).



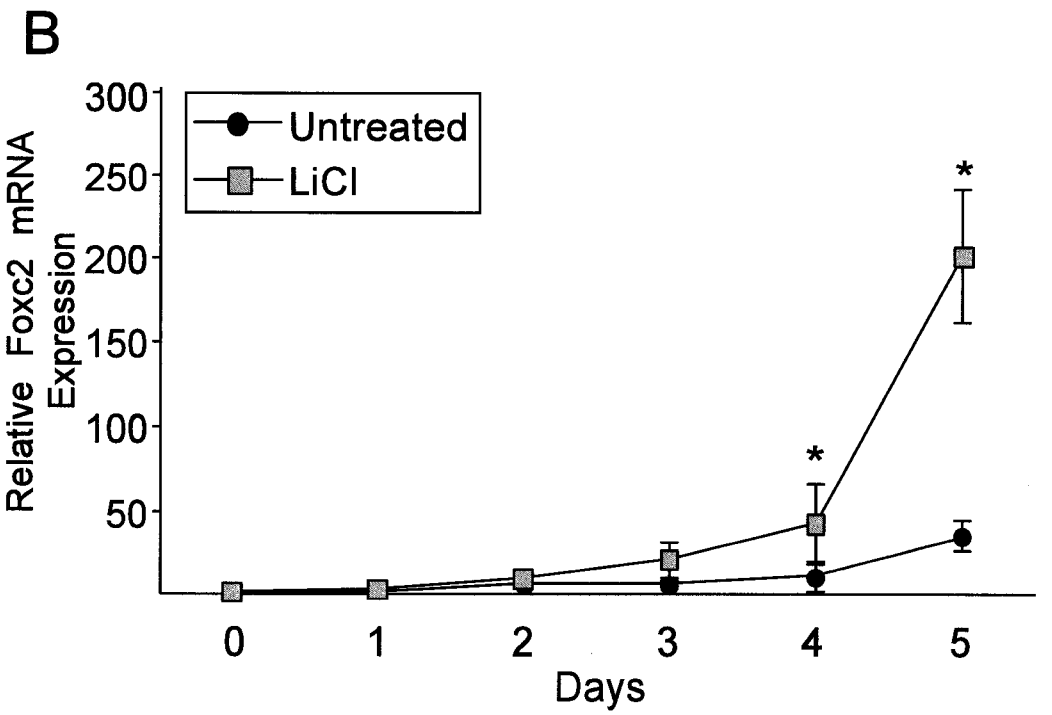
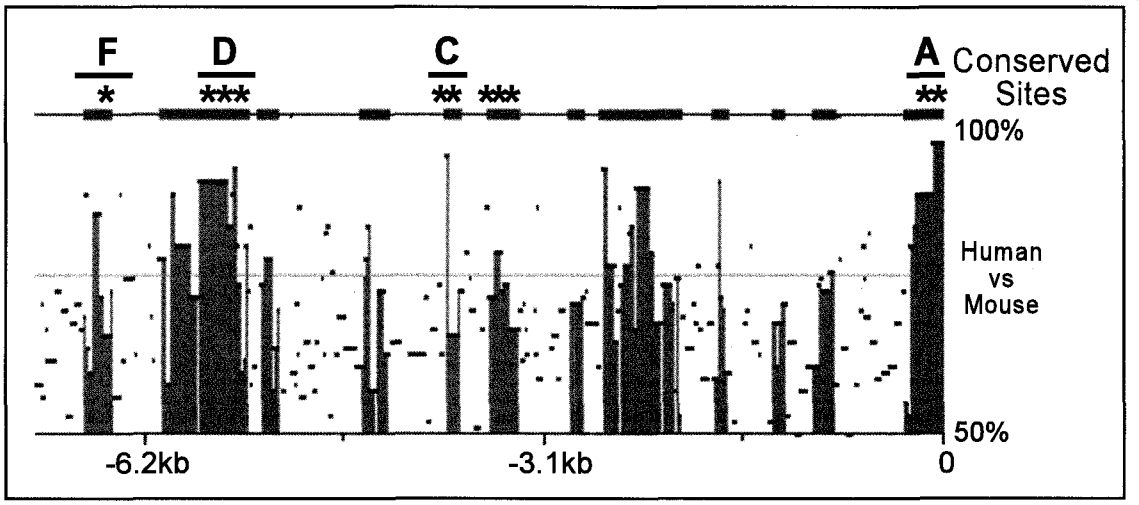
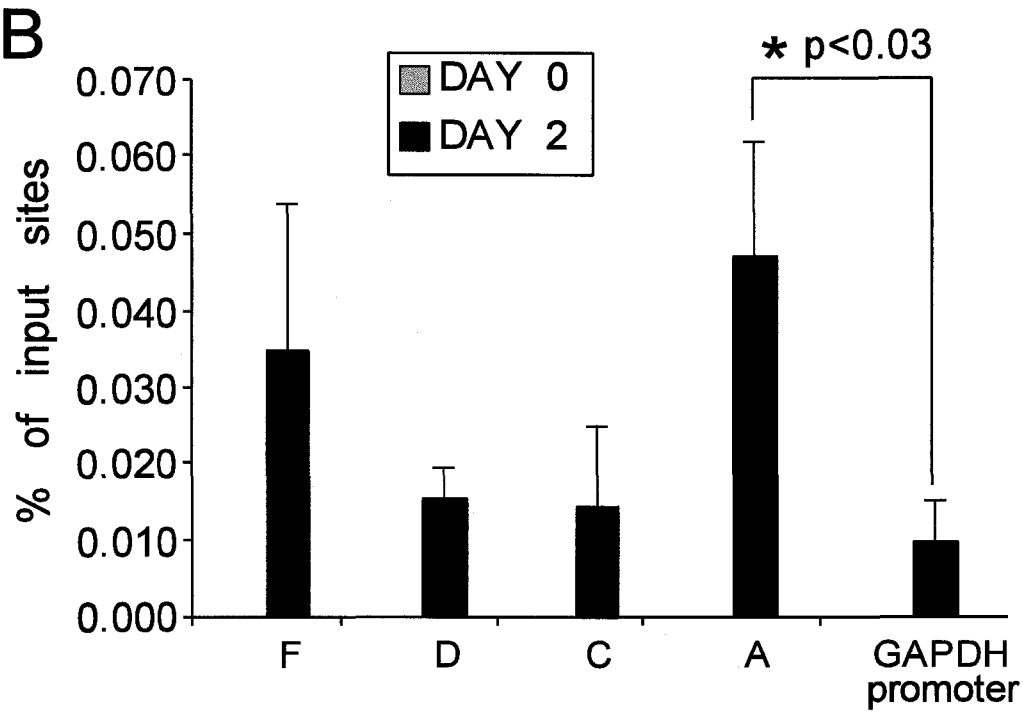


Figure 3.3 – β-catenin is present at conserved sequences upstream of the Foxc1 gene. Mouse genomic DNA sequences 25 kb upstream of the Foxc1 gene, along with the homologous region from the human genome, were entered into the multisequence local alignment tool MULAN (http://mulan.dcode.org) to identify conserved regions. Following sequence alignment, conserved binding sites were determined using multi-TF transcription factor binding site analysis. Several putative LEF/TCF binding sites were identified using this method, as marked by the asterix (*) on the alignment. To determine if β-catenin is enriched at these sites, chromatin immunoprecipitation (ChIP) was performed. Cross-linked nuclear extracts from day 0 and 2 of P19 cell differentiation were incubated with an anti-β-catenin antibody or pre-immune sera. Putative binding regions were amplified by real-time PCR. Binding to the non-related GAPDH promoter was used to determine background. Results were calculated and presented as the percentage of total available sites for immunoprecipitation that were actually bound by β-catenin. Statistical significance was determined using a one-way ANOV, *$p<0.03$.

A



B



3.3.4 Gli2 regulates Foxc1/2 expression

The Wnt signaling pathway has previously been identified as a regulator of Gli2 expression (8, 36, 42). To gain a better understanding of the pathways that cooperate to regulate Foxc1 and Foxc2 expression, we decided to look at the expression patterns of Foxc1/2 in the presence of a dominant-negative Gli2 during myogenesis in P19 cells. P19[Gli/EnR] cell lines were differentiated in the presence of DMSO and stained with an anti-myosin heavy chain antibody (Figure 3.4.1, A). Overexpression of Gli/EnR is inhibitory towards myogenesis (Figure 3.4.1, A and B), as previously reported by our lab (35). Northern blot analysis confirmed that P19[Gli/EnR] cells do not express MyoD and downregulate Meox1, as previously described (35). Analysis of Foxc1 transcript levels by real-time PCR and Northern blot indicates a 30% (± 12%, n=9) to 9% (± 7%, n=9) decrease in Foxc1 levels from days 3-6. Variability in the rate of Foxc1 loss was observed between the different P19[Gli/EnR] clones. However, Foxc1 expression was severely decreased by day 9 (6%± 4%) compared to P19[Control] cells, in all clones studied (Figure 3.4.1, C). Foxc2 expression on the other hand, is completely abolished (98% ± 1% decrease) as early as day 3 (Figure 3.4.1, C). These data suggest that there may be differential regulatory mechanisms by Gli2, or genes regulated by Gli2, on Foxc1 and Foxc2 expression patterns.

Having established that the loss of functional Gli2 leads to a down-regulation of Foxc1/2 expression, we performed gain-of-function studies to see if Gli2 is sufficient to drive Foxc1 and Foxc2 gene expression in aggregated P19 cells. P19 cells overexpressing Gli2 are able to differentiate into myocytes in the absence of DMSO, and also upregulate the expression of MyoD and α-actin (Figure 3.4.2, A and B), in agreement with previous results (35). Using real-time PCR, we detected a 6-fold (± 3-fold, n=2) increase in Foxc1

Figure 3.4.1 – Functional Gli2 is required for proper Foxc1/2 expression. P19[Control] and P19[Gli/EnR] cells were aggregated in the presence of DMSO for 9 days. Following differentiation, cells were fixed and immunofluorescence was performed using an anti-Myosin Heavy Chain antibody to detect myocytes and Hoechst dye to label nuclei (Panel A). Scale bar represent 20μm. For Northern Blot analysis, the membranes were hybridized with labelled cDNA fragments corresponding to the factors indicated on the right. Results shown are representative of three P19[Meox/EnR] cell lines (Panel B). Quantitative PCR and densitometry analysis of Northern blots were performed in order to quantify the amount of Foxc1 and Foxc2 transcripts present in these cell lines. Results, normalized to GAPDH or 18S are expressed as a percentage of control cells on the given day, and are shown as the average of three separate cell lines, from three differentiations, +/- SEM, *$p<0.001$ (Student's T-test) (Panel C).

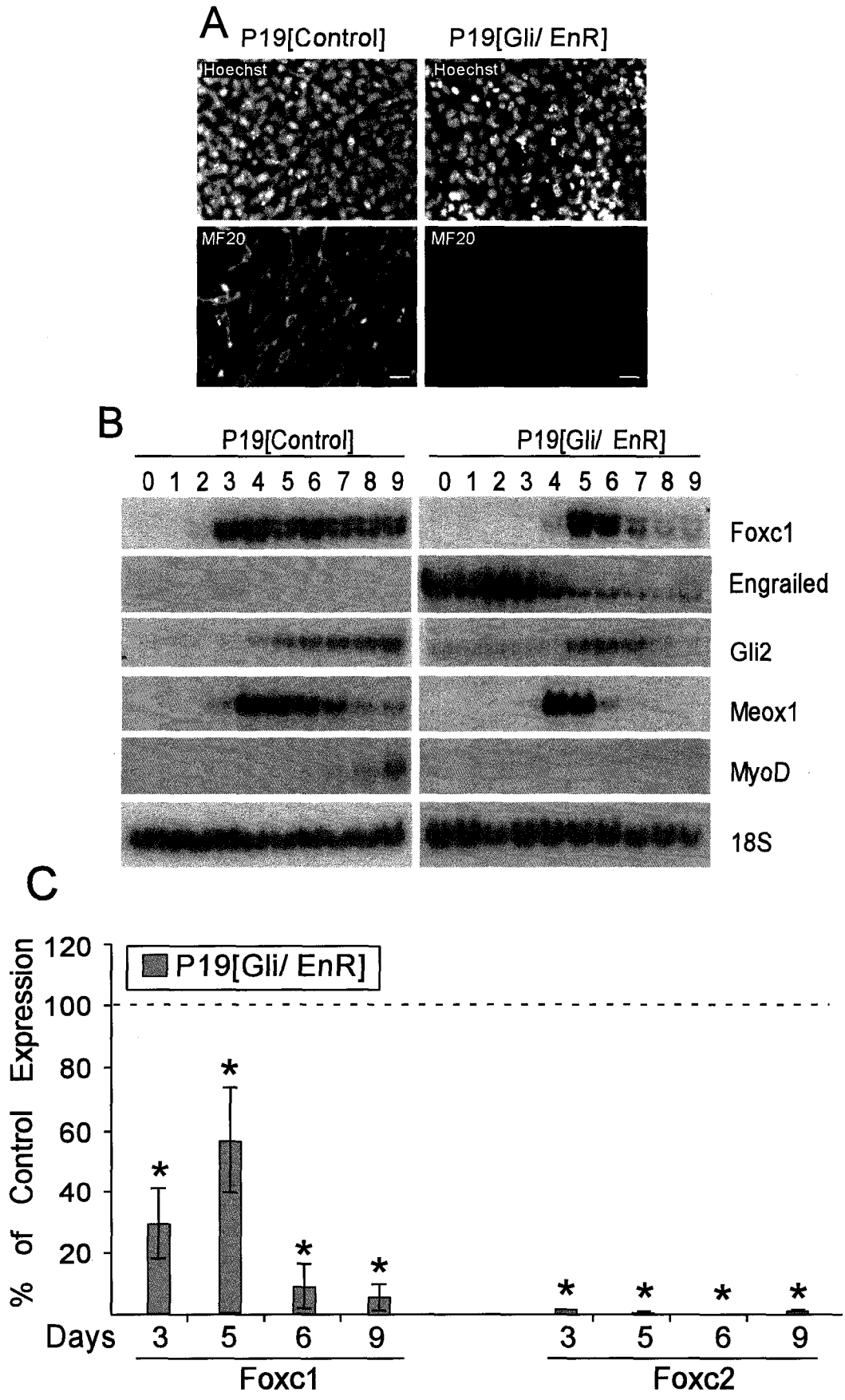
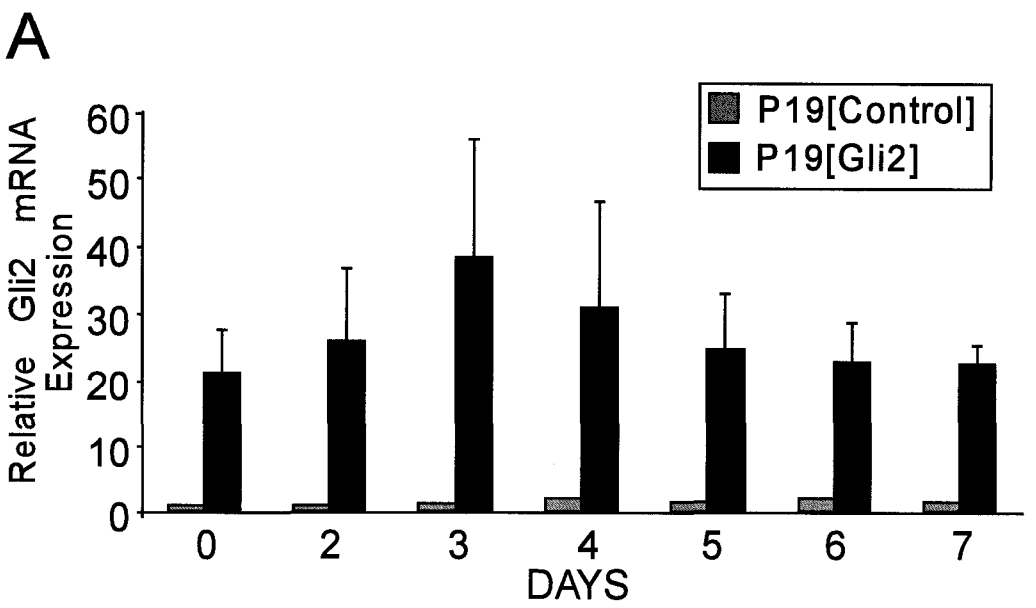
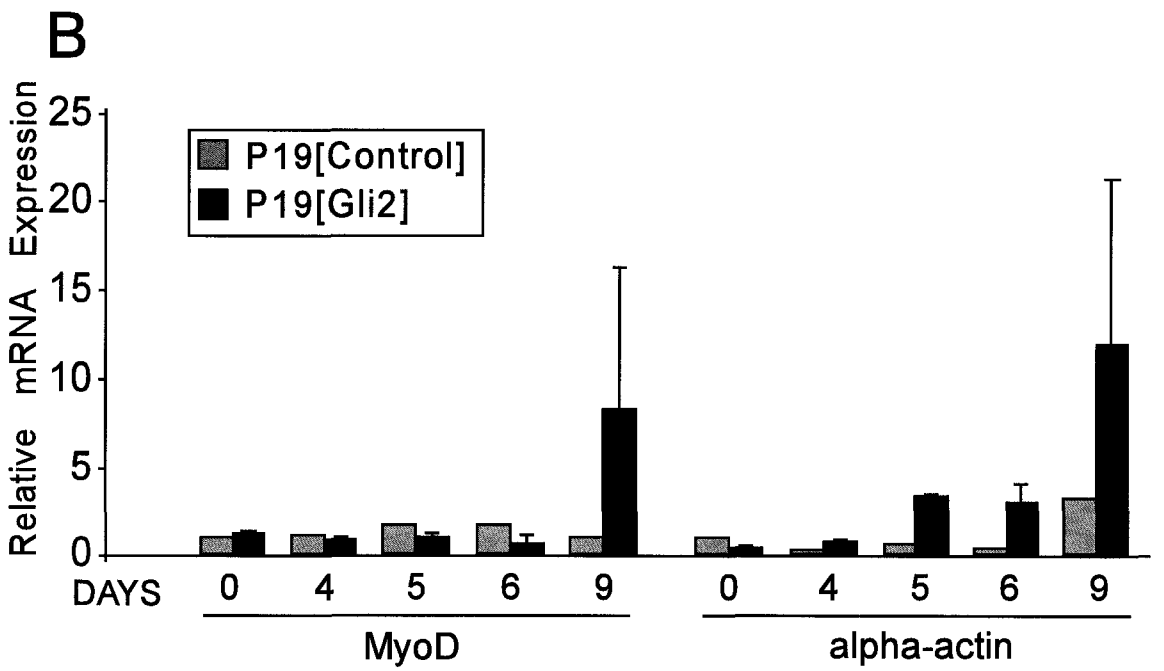


Figure 3.4.2 – Gli2 is sufficient to induce myogenesis in the absence of DMSO. P19[Control] and P19[Gli2] cells were aggregated in the absence of DMSO for 9 days and mRNA expression levels examined by real-time PCR. Results are shown normalized to GAPDH and relative to day 0 and are the average of three separate cell lines +/- SEM Examination of transcript levels confirmed that P19[Gli2] cells overexpress Gli2 (A) and have elevated levels of MyoD and α-actin (B).



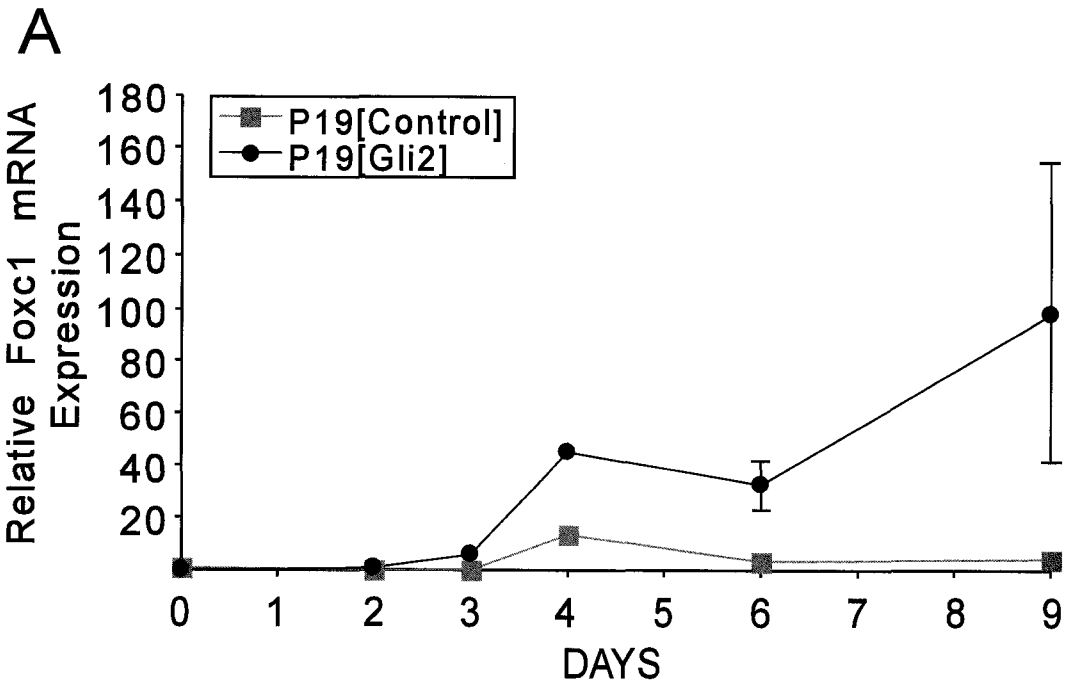


and a 182-fold (± 58-fold, n=2) increase in Foxc2 expression on day 3 of differentiation in P19[Gli2] cells, when compared to P19[control] cells (Figure 3.4.3, A and B). Both Foxc1 and Foxc2 remain upregulated throughout the remainder of the differentiation. These results suggest that Gli2 contributes, directly or indirectly, to the regulation of both Foxc1 and Foxc2 expression during skeletal myogenesis in P19 cells.

3.3.5 Meox1 contributes to the regulation of Foxc1/2 expression

Our lab has previously shown that forced Gli2 expression enhances Meox1 and induces MRF expression under non-muscle-inducing conditions (35). Based on these observations, we were interested in investigating the possibility that Meox1 can regulate expression of both Foxc1 and Foxc2. Ectopic expression of the Meox/EnR transcription factor results in impaired myogenesis, as shown by the lack of Myosin Heavy Chain-positive cells in the cultures (Figure 3.5, A). These cell lines, which fail to differentiate into skeletal myocytes in culture, also exhibit a decrease in Gli2 expression, as well as a complete absence of Pax3, endogenous Meox1 and MyoD transcripts (Figure 3.5, B), which is in accordance with published data (35). In differentiating P19[Meox/EnR] cells, there is a range from 78-100% loss of Foxc1 transcripts from Days 4-6 (Figure 3.5, C). Foxc2 transcripts also show a loss of 75-99% from days 4-6 when compared to P19[Control] cells. Therefore, the loss of Meox1 function leads to the downregulation of Foxc1/2 transcripts. The slight upregulation of Foxc1/2 transcripts later during the time course suggests that other factors are able to regulate Foxc1/2 gene expression to some extent.

Figure 3.4.3 – Gli2 induces both Foxc1/2 gene expression in the absence of DMSO. P19[control] and P19[Gli2] cells were aggregated in the absence of DMSO for 9 days and mRNA expression levels examined by real-time PCR. Results are shown normalized to GAPDH and relative to day 0 and are the average of three separate cell lines +/- SEM. Foxc1 and Foxc2 transcripts were upregulated in P19[Gli2] cell lines (A and B).



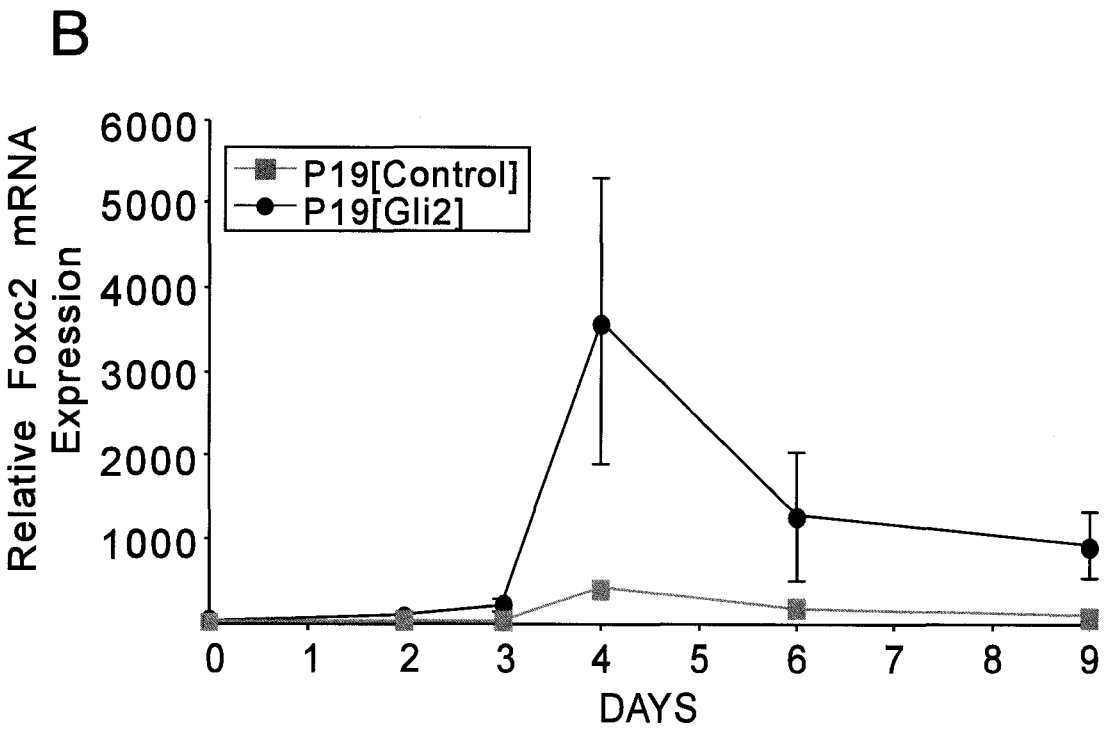
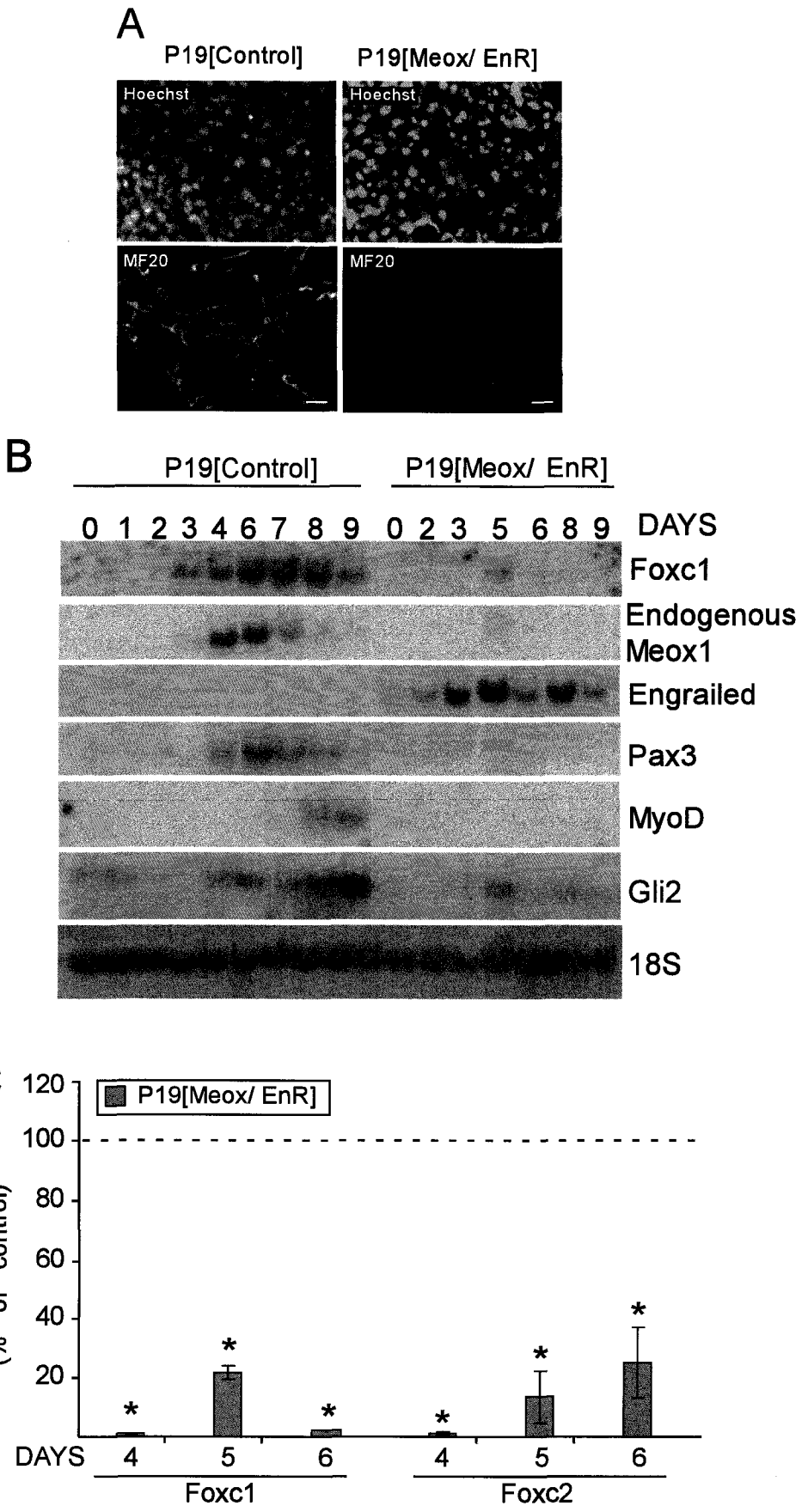


Figure 3.5 – Foxc1 and Foxc2 expression is decreased in the absence of functional Meox1. P19[Control] and P19[Meox/EnR] cells were aggregated in the presence of DMSO for 9 days. Cells were fixed on day 9, and immunofluorescence was performed using an anti-MHC antibody to detect myocytes and Hoechst dye to identify nuclei (A). Scale bar represents 20μm. For Northern blot analysis, the membranes were reacted with cDNA probes corresponding to the factors indicated on the right (B). Real-time PCR was used to measure the levels of Foxc1 and Foxc2 in these cell lines. Results shown were normalized to GAPDH, expressed as a percentage of control cells on the given day, and are the average of three separate cell lines +/- SEM. *$p<0.008$, compared to control group (Student's T-test) (C).



3.4 Discussion

The results of these studies have demonstrated that Foxc1 and Foxc2 transcripts are detected early, and in an overlapping pattern with Wnt3a, during P19 cell differentiation into skeletal muscle. These findings are consistent with their previously established role of regulating somitogenesis (27, 48). By investigating the effect of various dominant-negative transcription factors on Foxc1/2 transcription levels, we have shown that Foxc1 and Foxc2 are dynamically regulated during myogenesis in P19 cells. Specifically, we provide evidence that β-catenin directly regulates Foxc1 transcription, given that the presence of a dominant-negative β-catenin completely abolishes Foxc1 expression, LiCl upregulates Foxc1 expression, and that β-catenin occupies the Foxc1 promoter. To our knowledge it is the first evidence that these FOX genes are directly regulated by β-catenin. Our data are further supported by results which show that in the absence of either a functional Meox1 or Gli2 transcription factor, which lie downstream of ß-catenin in the Wnt signaling pathway, the expression of Foxc1/2 is severely decreased, compared to control cells. Taken together, these data suggest that the expression of Foxc1 during skeletal mesoderm patterning is regulated by the members of known networks controlling myogenesis.

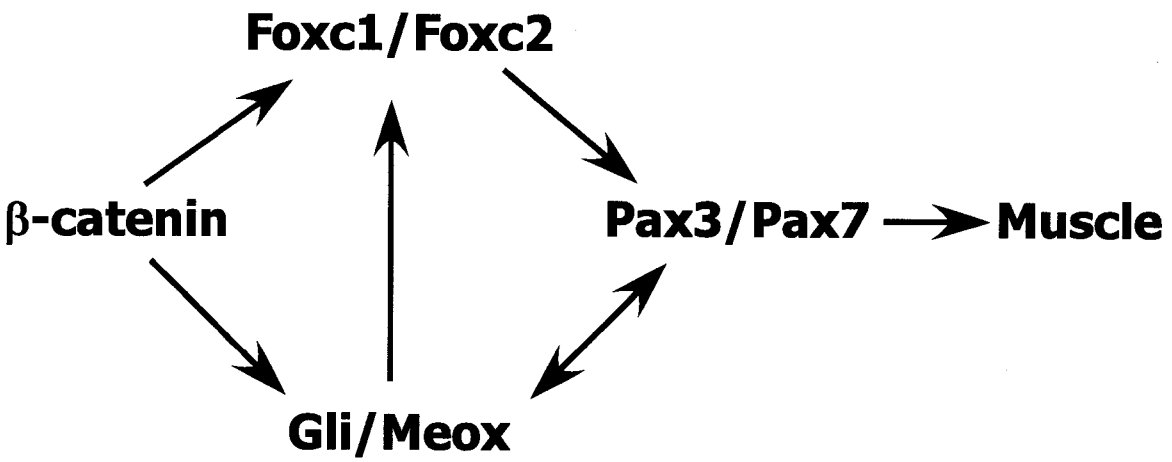
Wnt ligands have been implicated as playing an important role in muscle progenitor fate determination, terminal differentiation and muscle patterning (1, 7, 19, 22, 34, 46). Wnt1, secreted from the dorsal neural tube, induces myogenesis via the expression of Myf5, whereas Wnt6 and Wnt7a, emanating from the dorsal ectoderm activate MyoD expression (46). Wnt1/3a/4/8 have been shown to signal via the canonical pathway which ultimately results in the stabilization and accumulation of nuclear β-catenin, which in concert with the LEF/TCF family of factors, is capable of regulating downstream targets (17, 38, 40). It has

recently been proposed that β-catenin is capable of binding an early epaxial myogenic enhancer, and, in collaboration with Gli2/3, regulates Myf5 expression (6). Aside from its well-known role in regulating early myogenic events, canonical Wnt signaling has been shown by others to regulate the expression of Forkhead family members during various developmental processes, however no direct regulatory interaction of Fox members with β-catenin was reported. For example, Wnt 1 and 4, which are both known to utilize the canonical pathway, are necessary for the expression of FoxN1 during thymic development (4) whereas β-catenin is sufficient to induce expression of the endodermal genes Foxa1 and Foxa2 (43).

In our study, we show that there is overlap between the expression of Wnt3a and Foxc1 in differentiating stem cells. Therefore, it is possible that the induction of the canonical pathway by Wnt ligands during myogenesis results in the expression of Foxc1. Our ChIP data suggest that β-catenin occupies conserved regions of mouse genomic DNA proximal to the Foxc1 transcriptional start site. Although previous studies have identified direct β-catenin targets in cancer models, (54) (see also http://www.stanford.edu/~rnusse/pathways/targets.html for a complete list of known Wnt targets) this is, to our knowledge, the first observation of β-catenin binding to the promoter of a Fox gene family member. However, since the regulatory regions controlling Foxc1 expression in the early somites have not been identified and verified *in vivo*, more work is needed to validate these regions as *bona fide* regulatory regions. It is also possible that enhancers exist in other distal locations that may also bind to β-catenin. These experimental findings suggest that the Wnt/β-catenin pathway is necessary for the initial activation of Foxc1 expression in differentiating P19 cells.

While β-catenin seems to be necessary for the initial expression of Foxc1/2, our data also demonstrate that in the presence of dominant-negative Meox1 and Gli2 transcription factors, Foxc1/2 expression is severely decreased. Since both endogenously expressed Gli2 and Meox1 are first detected following Foxc1/2 onset, they are most likely involved in maintaining Foxc1/2 expression during differentiation. Both Gli2 and Meox1 have been shown to function in a positive regulatory loop which is essential for myogenesis to occur (35). In addition, Pax3, Meox1 and Gli2 appear to function downstream of β-catenin during P19 cell differentiation (36). Others have also shown that the canonical Wnt pathway regulates Gli2 expression in the developing embryo (8, 42). However, Gli2 is a known downstream effector of the Shh pathway involved in regulating the expression of muscle-specific genes (6, 32). Thus, there exists the possibility that both Wnt and Shh signaling pathways cooperate to regulate the expression of Foxc1 and Foxc2 during myogenesis. It is possible that once activated, the Meox1-Gli2 regulatory loop is responsible for maintaining Foxc1 expression (Figure 3.6). In addition, Foxc1/2 compound homozygous mice display a decrease in the levels of Meox1 transcripts in the paraxial mesoderm and somites, suggesting that Meox1 is a downstream transcriptional target of Foxc1, thereby placing Foxc1 within the Meox1-Gli2 regulatory loop. It is interesting to note however, that even in the presence of a dominant-negative Meox1 or a dominant-negative Gli2, Foxc1 transcripts are still detectable, and yet myocyte differentiation does not occur. It is possible that under these circumstances, a necessary Foxc1 co-factor is not expressed, or that Meox/EnR and/or Gli/EnR are bound to the regulatory regions of genes involved in terminal differentiation. This would lead to chromatin-remodeling and ultimately, to the prevention of future

Figure 3.6 – Working Model. Working model for the role of Foxc1 within the network of transcription factors which regulate skeletal myogenesis in P19 cells. Red arrows indicate the findings of this study, while black arrows represent published results from other labs (12, 16, 27, 30, 31, 35, 36, 39, 41, 49, 51)



expression from these promoters, even in the presence of positive-acting transcription factors.

In summary, we provide evidence that Foxc1 and Foxc2 expression is initially reliant on canonical Wnt signaling, whereas Foxc1 appears to be part of a regulatory loop consisting of Meox1 and Gli2, which is important for sustaining the expression of Foxc1 during the later stages of myogenesis. This study furthers our fundamental understanding of the molecular mechanisms that govern lineage determination and cell fate specification in P19 EC stem cells.

3.5 Acknowledgments

The authors would also like to thank Karen Kennedy for critically reading this manuscript. We gratefully acknowledge the financial support of the Canadian Institutes of Health Research (CIHR), Institute of Aging in the form of a Studentship to J.S. I.S.S. was supported by a Canadian Institute of Aging Investigator Award. This work was funded by a CIHR grant (MOP-84458) to I. S. S.

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Chapter 4

Canonical Wnt Signalling and Retinoic Acid Regulate Sox7 Expression

4.1 INTRODUCTION

The Wnt family of signalling molecules is essential for regulating a multitude of developmental processes (35, 46). The canonical Wnt pathway is initiated by binding of the Wnt ligands to the Frizzled (Fz) cell surface receptors, initiating a cascade of signalling events that ultimately results in the regulation of target gene expression by β-catenin (3). Characterization of *Fz* expression in the developing mouse embryo reveals a dynamic expression pattern. Fz1, Fz6, Fz7 are expressed in newly formed somites, whereas Fz9 is only detectable at later stages in the myotome of E10.5 embryos (5, 39), implying a role for Wnt signalling during muscle development. In fact, Wnt1,-3a and -4 are expressed in the dorsal half of the neural tube as epaxial myogenesis begins (6, 18, 31, 34), and ectopic expression of these Wnts in chick results in the expansion of the epaxial muscle domain at the expense of the sclerotome (43). Ectopically expressed Wnt1, 3a, 4 and 6 have also been shown to increase and expand the expression domain of Pax3 and Pax7 within the developing somite (33). An essential role for Wnts has been identified by examining muscle development in knockout mice. Mice null for either Wnt1 or Wnt3a show a loss of the medial compartment of the dermomyotome (DM), as well as a reduction in Pax3 and Myf5 expression (14, 19).

The process of myogenesis and somite patterning can also be influenced by the presence of the Vitamin A derivative retinoic acid (RA). RA is synthesized locally first by conversion of retinol to retinaldehyde which is further processed to RA by the retinal dehydrogenase (Raldh) class of enzymes (8). Studies in knockout mice reveal that three members of the Raldh family, Raldh1-3, control most of the embryonic RA synthesis (9, 12, 27, 28, 32). Raldh2, first expressed at E7.5 in the paraxial mesoderm is responsible for all signalling activity up to E8.5, whereas Raldh1 and Raldh3 exert their effects at later stages

during development of the eye, olfactory pit and kidney (26-29). A balance in the expression of these RA-synthesizing enzymes with RA-degrading enzymes generates RA gradients within the embryo, establishing the anterior-posterior axis (1, 4, 32, 38), and determining left-right asymmetry during somitogenesis (40-42). In addition to its role in regulating somite patterning, RA positively regulates myogenesis by increasing expression levels of the MRFs and enhancing overall muscle development in stem cells (2, 11, 13, 17, 30). Retinoid signalling is achieved by binding of the RA ligand to the nuclear retinoic acid receptor (RAR) or the retinoid X receptor (RXR). Heterodimers of these two receptors bind to conserved retinoic acid response elements (RARE) in the promoter of genes and positively regulate gene expression (7, 15, 16, 23, 24).

Both Wnt signalling and RA have been shown to regulate myogenesis in the P19 cell model (36, 37 and K. Kennedy, unpublished results). Overexpression of Wnt3a in P19 cells is sufficient to induce the entire myogenic program, while expression of Wnt3a in P19[MyoD] or P19[Mgn] cell lines activates MRF function and leads to an increase in the number of MHC-positive cells (37). Expression of an activated form of β-catenin also induces myogenesis, and expression of a dominant-negative β-catenin mutant abolished skeletal muscle development (36). Taken together, these results suggest that Wnt3a regulation of myogenesis in P19 cells occurs via activation of β-catenin in the canonical Wnt pathway. Treatment of P19 cells with retinoic acid accelerates and enhances skeletal myogenesis while preventing differentiation of cardiac muscle cells. It has also been demonstrated that RA-mediated myogenesis causes an increase in Wnt3a expression, as well as in Sox7 expression (K. Kennedy, unpublished results). Sox7 is a member of the large Sox family of transcription factors. It is expressed throughout the somite of E7.5-8.0 mice, and

has been shown to determine the fate of mesodermal derivatives by regulating the expression of mesoderm-inducing genes in Xenopus (47). Our lab has previously identified a novel role for Sox7 in regulating skeletal myogenesis under conditions which do not normally support differentiation (Savage, thesis chapter 2). Studies in P19 cells demonstrate that Sox7 expression precedes that of the muscle precursor markers, making it a potential target of both Wnt and RA signalling. In this study, we aim to further characterize the ability of both the canonical Wnt signalling pathway and RA to modulate Sox7 expression, as well as the relationship and interplay between the two pathways.

4.2 Materials and Methods

4.2.1 Cell Culture

P19 EC cells (ATCC CRL-1825) were maintained in alpha-minimal essential media as previously described (45). Differentiation was induced by aggregating cells in suspension in the presence of 1% DMSO for 4 days before transferring them to tissue culture dishes for an additional 5 days. For the experiments involving treatment with retinoic acid (RA), the cells were either treated with 1% DMSO, or with 1% DMSO and 0.5 nM all-trans retinoic acid (Sigma-Aldrich, R-2625) during the initial four days of cellular aggregation in suspension. To activate the canonical Wnt signalling pathway, cells were aggregated and either left untreated (control) or treated with 20mM LiCl during the entire 4 days of aggregation. Treatment of cells with LiCl in order to induce canonical Wnt signalling has been described (20). The P19[β-catenin/EnR] cell lines have been generated by our lab as previously characterized (36). All differentiations were performed three independent times.

4.2.2 Reverse Transcription and Quantitative PCR

We generated cDNAs using the Quantitect Reverse Transcription kit (Qiagen, Mississauga, ON), from 1μg of RNA isolated using the RNeasy mini kit (Qiagen, Mississauga, ON). For real-time detection of mRNA expression, we used $1/20^{th}$ of the total first strand synthesis product as a template for PCR amplification using FastStart SYBR Green with ROX (Roche Applied Sciences, Laval, Québec). All reactions were performed and analyzed using the SDS Software of the ABI 7300 (Applied Biosystems, Streetsville, ON, Canada). Primer sequences used in these studies have been provided in Appendix A. Relative gene expression was calculated using the comparative Ct method as previously described (22). All reactions were performed in duplicate, and the results shown are the average +/- SEM of three independent experiments.

4.2.3 Chromatin Immunoprecipitation (ChIP)

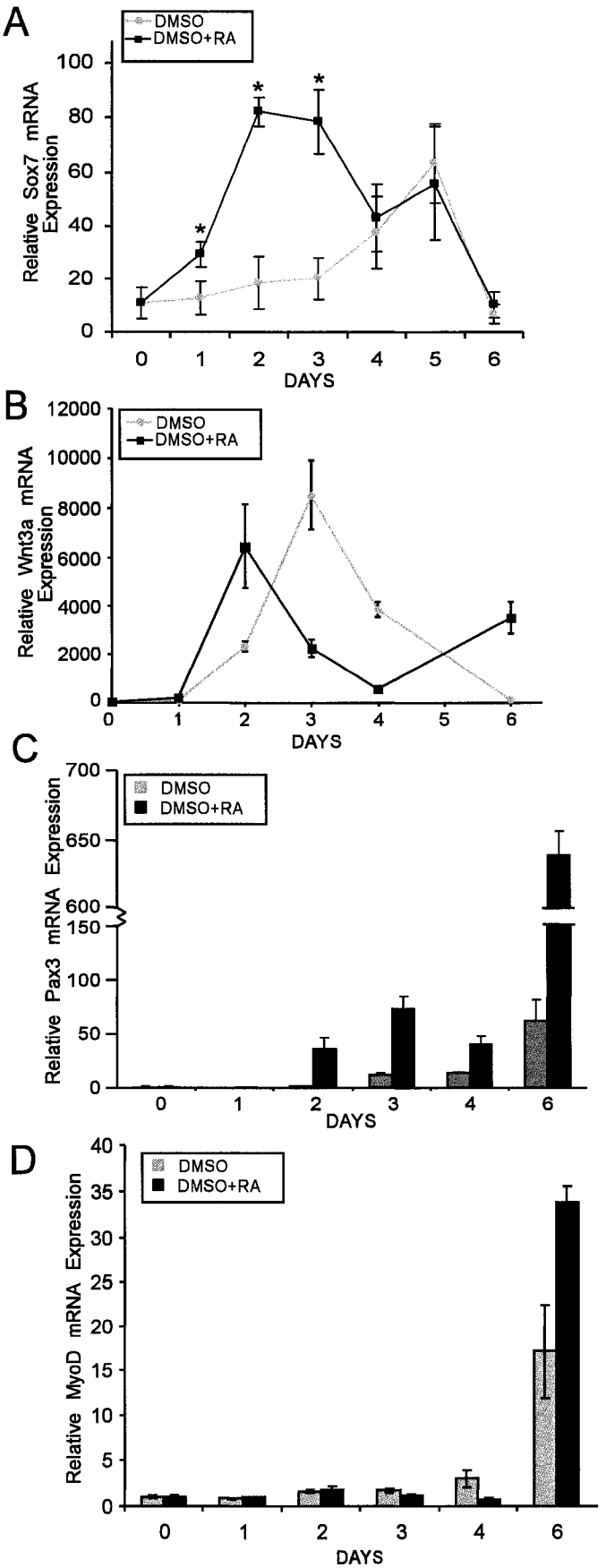
P19 cells were differentiated for two days in the presence of DMSO, or DMSO+RA and processed according to the methods described in Chapter 2, section 2.2.7, prior to incubation with 5μg of β-catenin antibody (clone 15B8, Sigma-Aldrich) or 5μg of IgG antiserum (Zymed Laboratories, California). Samples were treated with 20μg Rnase A and 40μg Proteinase K and DNA purified using Qiagen's PCR Purification Kit (Qiagen, Mississauga, ON). Enrichment of binding sites was analyzed using SYBR Green real-time PCR, as described above. Please refer to the Appendix for the sequence of primers used in this experiment.

4.3 RESULTS

4.3.1 Retinoic acid precipitates the expression of Sox7 and Wnt3a

Our lab has previously demonstrated that treatment with a range of RA concentrations from 0.5 to 30nM is sufficient to enhance and accelerate skeletal myogenesis, while simultaneously suppressing the cardiac myoblast cell fate (Kennedy et al., unpublished data). In accordance to the studies of Kennedy et al. (unpublished observations), we showed that Sox7 is an RA-responsive gene, whose expression peaked at 82 ± 5% of maximum (n=5) on day 2 of differentiation in RA-treated cells, compared to the 19 ± 10% (n=5) of maximum observed in cells treated with DMSO only (Figure 4.1, A). Furthermore, Wnt3a transcript expression was accelerated upon treatment of cells with RA, which coincided with the onset of Sox7 expression. In this case, Wnt3a peaked on day 2 following RA stimulation, a day earlier than the maximal peak observed on day 3 for cells treated with DMSO only (Figure 4.1, B). It was interesting to note that both Sox7 and Wnt3a were upregulated on day 1 whereas Pax3 was not upregulated until day 2 (Figure 4.1, C), suggesting that these two factors may act upstream of Pax3 during myogenesis in P19 cells. Finally, to confirm that the changes in gene expression observed following RA treatment were occurring in the myogenic pathway, we assessed the levels of MyoD transcripts using real-time PCR. Treatment of cells with 0.5 nM RA caused a 34-fold (±2, n=3) fold change in MyoD expression, compared to the 17-fold (±5, n=3) change observed in control cells (Figure 4.1, D). These results suggest that both Sox7 and Wnt3a may lie downstream of retinoic acid signalling during skeletal muscle differentiation in P19 EC cells. Our studies here provide a more detailed analysis of Wnt3a and Sox7 expression in the presence of RA. Given the

Figure 4.1 – Retinoic acid enhances the expression of Sox7 and Wnt3a. P19 cells were differentiated in the presence of 1% DMSO or DMSO+0.5nM retinoic acid, and RNA harvested from days 0-6. Q-PCR analysis of gene expression was used to measure the relative mRNA levels of Sox7 (A), Wnt3a (B), Pax3 (C) and MyoD (D). Changes in gene expression patterns were calculated using the comparative Ct method (22). Results were normalized against the internal control GAPDH, and are expressed as a percentage of maximum expression observed for each differentiation. Bars represent average ± SEM, n=5 independent differentiations for Sox7, and average ± SEM of three technical replicates from one differentiation for panels B-D. Statistical analysis versus control at each time point was carried out using Student's T-test (*$p<0.05$).



observation that Wnt3a is upregulated prior to the onset of Sox7 transcription, we sought to further investigate the relationship between RA and Wnt signalling on Sox7 expression.

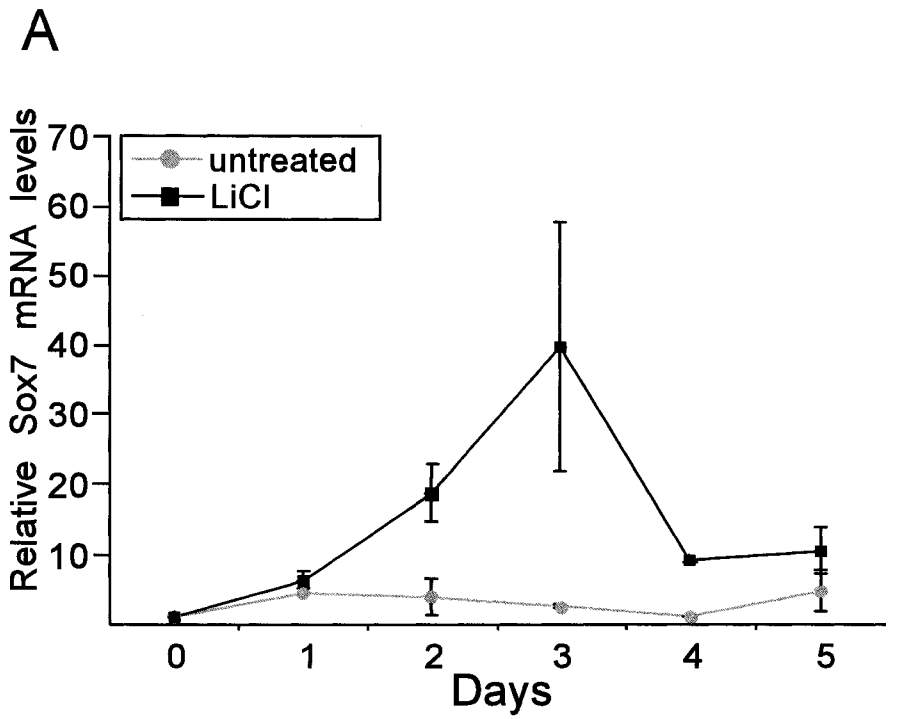
4.3.2 Canonical Wnt signalling is important for Sox7 expression

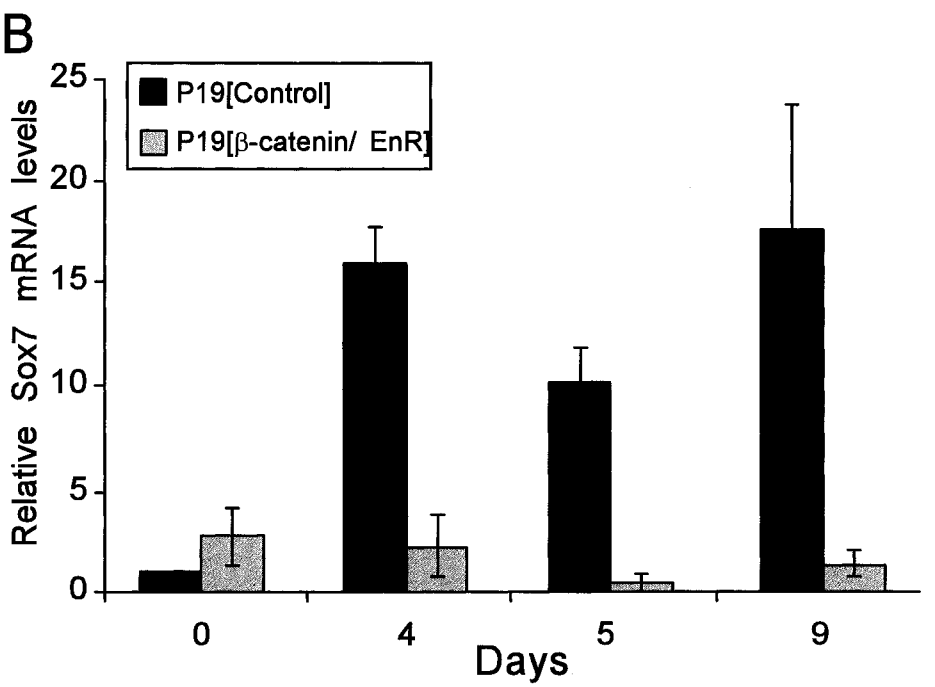
To further investigate the possibility that Wnt signalling regulates Sox7 expression during myogenesis, P19 cells were aggregated and either left untreated, or were treated with 20mM LiCl, a glycogen synthase kinase 3 (GSK3) inhibitor that mimics active Wnt signalling (20). Cells aggregated in the absence of LiCl did not upregulate Sox7 to any appreciable levels, as shown in Figure 4.2, Panel A. However, the addition of LiCl was sufficient to enhance Sox7 mRNA levels 6-fold (±1, n=3) on day 1 compared to 4-fold (±0.1 fold, n=3) for untreated cells. Sox7 expression continued to increase upon LiCl stimulation, peaking at 40-fold (±18, n=3) on day 3, compared to control cells (2±0.2, n=3) (Figure 4.2, A). Having established that induction of the canonical Wnt pathway is sufficient to cause an increase in Sox7 mRNA expression, we performed the converse experiment, where we measured Sox7 expression levels in the presence of a dominant-negative β-catenin.

4.3.3 RA cannot completely rescue the inhibition of Sox7 expression by a dominant-negative β-catenin

So far, we have provided evidence that both the retinoic acid and Wnt signalling pathways are able to enhance Sox7 expression in differentiating P19 cells. Based on these observations, we were interested in determining if the two pathways act genetically upstream of each other, or whether they act in parallel to modulate Sox7 mRNA levels. P19[Control] and P19[β-catenin/EnR] cell lines were differentiated in the presence of DMSO alone, or

Figure 4.2 – Sox7 expression is modulated by the canonical Wnt signaling pathway. Panel A: P19 cells were aggregated and either left untreated or treated with LiCl. Changes in gene expression were normalized against GAPDH and are expressed relative to Day 0 expression levels. Results shown are the average ± SEM of three independent differentiations. Panel B: P19[Control] and P19[β-catenin/ EnR] cells were differentiated in the presence of DMSO, and Sox7 expression levels were analyzed by Q-PCR. Results have been normalized to the endogenous control GAPDH and are expressed relative to P19[Control] day 0 levels.





DMSO+RA, and gene expression was analyzed using Q-PCR. Treatment of P19[Control] cell lines with DMSO+RA caused a 15-fold (±10, n=3) enhancement in Sox7 levels on day 4, compared to a 1-fold (±0.1, n=3) change observed in P19[β-catenin/EnR] cells that had received the same treatment (Figure 4.3). Therefore, it seems that the ability of RA to enhance Sox7 expression is dependent on β-catenin, or genes that are bound by β-catenin.

4.3.4 β-catenin associates with conserved sequences of the Sox7 regulatory region

While overexpression of a dominant-negative β-catenin transcription factor provides evidence that the Wnt signalling pathway is required for proper Sox7 expression, it does not demonstrate whether β-catenin is exerting its effects by directly binding to the Sox7 regulatory regions. To tackle this question, we employed the ChIP method to detect promoter occupancy by β-catenin. P19 cells treated with DMSO were aggregated for two days prior to harvesting the chromatin. Following incubation of cell extracts with either an antibody against β-catenin or pre-immune sera, the immunoprecipitated DNA was amplified using primer pairs that flank the two conserved LEF/TCF binding sites (LBE1 and LBE2) within a 4kb region of genomic DNA upstream of Sox7. These binding sites, identified using MuLAN, are conserved between the human and mouse genome (Figure 4.4, A). We determined that 0.025% (±0.007%, n=7) of input sites of day 2 cells treated with DMSO were bound by β-catenin at the LBE1 site, compared to 0.008% (±0.007%, n=7) at the LBE2 site and 0.002% (±0.001%, n=7) at the Pax3 promoter, which also contains evolutionarily conserved LEF/TCF sites (Figure 4.4, B). The enrichment of immunoprecipitated sites at the LBE1 region was statistically significant when compared to the enrichment at LBE2 or the

Figure 4.3 – Dominant-negative β-catenin inhibits RA-mediated induction of Sox7. P19[Control] and P19[β-catenin/ EnR] cells were differentiated in the presence of DMSO alone or DMSO + RA and assessed for Sox7 expression using Q-PCR. Fold gene expression was calculated using the comparative Ct method by normalizing the values to GAPDH. Results are expressed relative to P19[Control] day 0 expression, and bars represent average ± SEM for three independent experiments.

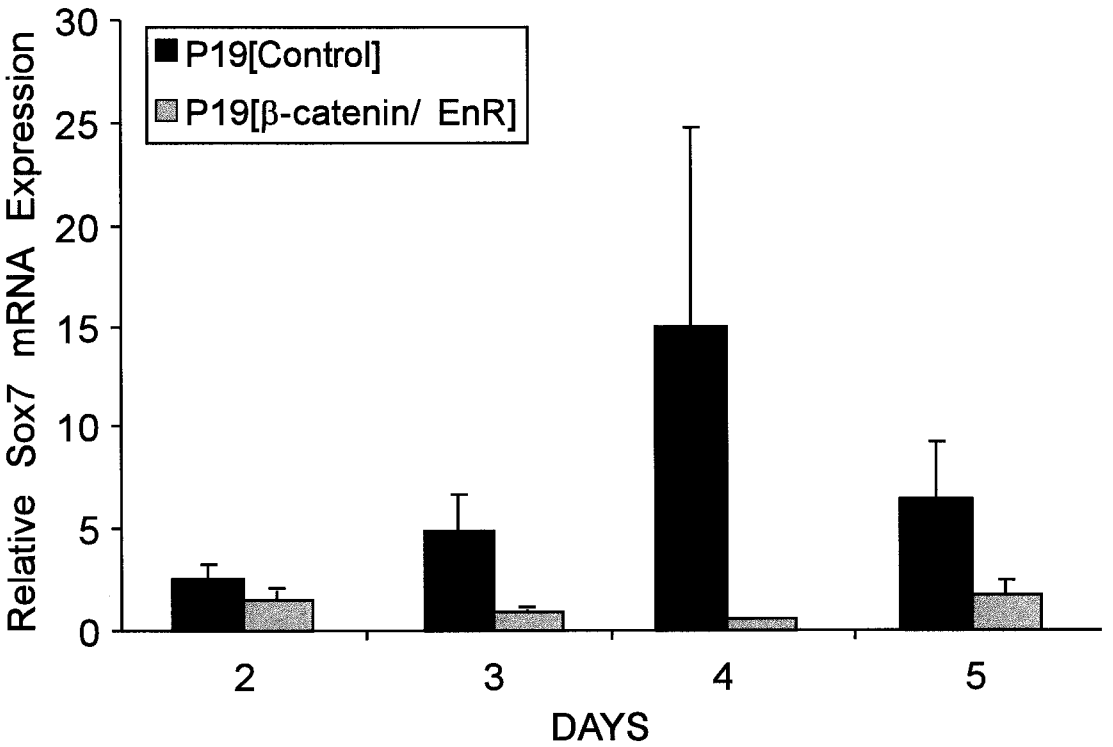
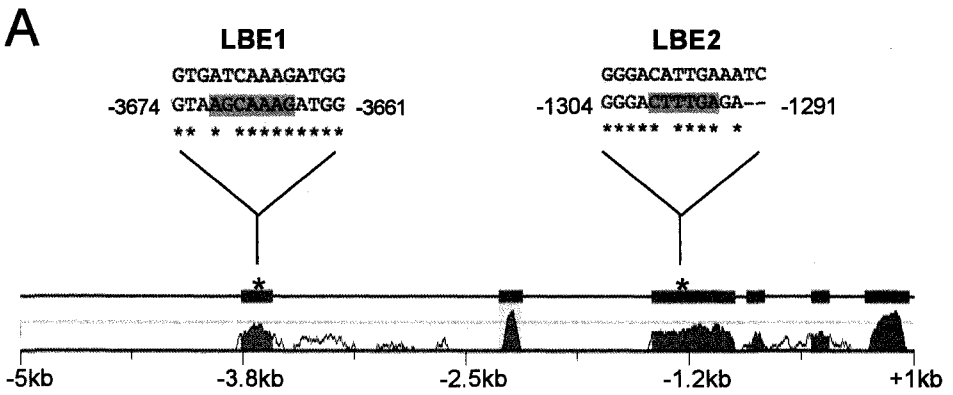
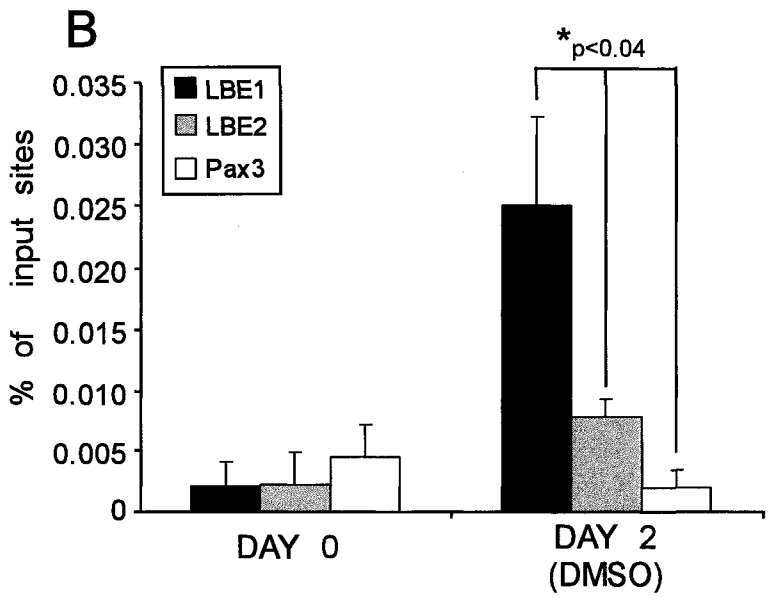


Figure 4.4 – β-catenin associates with conserved region upstream of Sox7. Panel A: Conserved lef/tcf binding elements (LBE) were identified by aligning DNA sequences from the mouse and human genomes using MULAN (mulan.dcode.org). Panel B: Cross-linked chromatin was isolated from P19 cells on day 2 of DMSO-induced differentiation. Immunoprecipitation was performed using an anti-β-catenin antibody or mouse IgG as a negative control. Amplification and quantification of target sequences were performed using SYBR-Green Q-PCR. Results are shown as percent of input sites that were immunoprecipitated. The Pax3 promoter contains an evolutionarily conserved LBE, and is used as a control region in this case. Bars represent the average ± SEM of four independent experiments. *$p<0.04$, one-way ANOVA.





Pax3 locus ($p<0.04$), and indicates that β-catenin is bound to endogenous Sox7 regulatory regions in a population of aggregated P19 cells.

4.4 Discussion

In this study, we have identified Sox7 as a novel retinoic-acid responsive gene, which is upregulated following exposure to the chemical. Furthermore, we demonstrate that treatment of P19 cells with RA can also modulate Wnt3a expression during myogenic differentiation, and that in turn, the canonical Wnt signalling pathway plays a significant role in regulating the expression of Sox7. Treatment of P19 cells with the GSK3β inhibitor LiCl is sufficient to induce expression of Sox7 transcripts, which are lost in the presence of a dominant negative β-catenin mutant. Furthermore, RA does not override the loss of Sox7 in the presence of the dominant-negative β-catenin. Finally, β-catenin binds directly to a conserved site in the regulatory region of the Sox7 gene. Taken together, using gain- and loss-of function approaches and ChIP, β-catenin appears to directly regulate Sox7 expression.

It has long been demonstrated that RA-stimulated EC cells undergo accelerated and enhanced myogenesis (11). Recently, our lab has demonstrated that RA-treated cells upregulate the expression of Pax3 and Meox1, genes which are important in the specification of the muscle precursor cell fate (Karen Kennedy, unpublished observations). Sox7 is also sufficient to enhance myogenesis in P19 cells by upregulating the expression of Pax3, Meox1 as well as the MRFs (Savage, thesis chapter 2). It is interesting to speculate, given the fact that we have identified Sox7 as an RA-responsive gene in this study that Sox7 may be partly

responsible for mediating the changes in expression of muscle precursor genes brought on by RA.

We have demonstrated in this paper that RA appears to act upstream of Wnt3a and cannot enhance Sox7 expression in the presence of a dominant negative β-catenin. However, we cannot disregard the possibility that the RA-bound RAR/RXR heterodimer may bind to the regulatory regions of the Sox7 gene during myogenesis, but are prevented from positively regulating transcription in the presence of the dominant-negative β-catenin. Therefore, based on our observations, we cannot positively conclude that RA acts genetically upstream of Wnt signalling. Using bioinformatical tools, comparative analysis of genomic DNA sequences from both the mouse and human genome has identified one evolutionarily conserved retinoic-acid response element (RARE) approximately 25kb upstream of the Sox7 transcriptional start site. The possibility that the RAR occupies the chromatin of the Sox7 gene is currently being investigated by other members of our lab. Further studies are also needed to verify whether region has any gene regulatory function for Sox7 gene expression.

In our cell model, treatment with the retinoid all-trans retinoic acid also induced expression of Wnt3a transcripts. These results are supported by observations that Wnt3a is induced during the self-renewal of RA-treated mES cells, and that this effect can be counteracted by β-catenin RNAi (25, 44). Studies in the F9 EC cell model provide additional evidence supporting the notion that an intact Wnt signalling pathway is essential for proper response to RA signals. RA has been shown to increase LEF/TCF sensitive transcription as early as 1 hour post-treatment, and a dominant-negative Tcf4 mutant is sufficient to prevent activation of RA-induced, TCF-dependent transcription and subsequent formation of primitive endoderm in F9 teratocarcinoma cells (21). It is also possible that

RA-induction of the canonical Wnt pathway serves as a negative-feedback mechanism, downregulating the Wnt signal. It has been demonstrated that RAR can compete with TCF for β-catenin binding, thus blocking the ability of β-catenin to activate target genes (10). Taken together, these results suggest that retinoic acid and the Wnt signalling pathways are intricately connected and work together to regulate gene expression during embryonic developmental processes.

In summary, the data presented here have provided insight into the regulatory mechanisms which control RA-mediated induction of myogenesis in P19 cells. We propose a model where the retinoic acid pathway works upstream of Wnt3a to regulate Sox7 expression, which ultimately results in activation of the entire myogenic program, as demonstrated in Chapter 2.

4.5 - ACKNOWLEDGMENTS

The authors wish to acknowledge the financial support from the following sources: J.S. was supported by a Canadian Institute of Health Doctoral Research Award (Institute of Aging). I.S.S. was supported by a Canadian Institute of Aging Investigator Award. This work was supported by grant MOP-49605 (to I. S. S.) from the Neuromuscular Research Partnership, an initiative of ALS Canada, Muscular Dystrophy Association of Canada, and the Canadian Institutes of Health Research.

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Chapter 5 – Discussion

5.1 – Proliferation Versus Differentiation of Muscle Precursor Cells

There has been considerable interest in using embryonic stem cells to replace damaged and dystrophic muscle. However, injection of ES cells into animals results in tumour formation and the percentage of cells that spontaneously differentiate into skeletal muscle are extremely low (48). Approaches where a population of mesodermal or muscle progenitor cells were purified prior to injection into damaged muscle have proven somewhat successful strategy for rebuilding dystrophic muscle tissue of mice (1, 4, 11, 43). However, differentiation is not one hundred percent efficient in these cases, and only small areas of muscle can be replenished. Finding efficient ways to deliver the cells to target tissues and dealing with the elicited immune responses have also impeded advances in the field of stem cell therapy. Although these problems are currently very active areas of research, this discussion will focus on the specification and differentiation of stem cells. Therefore, the goal of this research project was to further elucidate and understand the molecular mechanisms that regulate the process of myogenesis in P19 cells. The insight gained from the studies presented in this thesis may provide new targets and strategies for isolating and expanding the myogenic population prior to differentiation.

In Chapter 2, we identified a novel role for Sox15 in regulating pre-skeletal mesoderm formation in P19 EC cells. Sox15, when ectopically expressed in aggregated P19 cells was sufficient to induce the expression of Pax3/7, Meox1 and Foxc1, genes which are expressed in the PSM or DM during mouse embryonic development (5, 16, 19, 22, 30, 50). Sox15, however, was unable to induce the expression of MyoD, Myogenin or MHC. Interestingly, Myf5 transcripts were upregulated in the presence of Sox15, under conditions that normally do not support skeletal myogenesis. Myf5 has been reported as being the primary master regulator of skeletal muscle commitment (25). Moreover, Pax7 is required

for the direct expression of Myf5 and commitment of these cells to the myogenic lineage (28). A population of Pax3/Pax7-positive cells, originating from the central DM and persisting in skeletal muscle masses throughout fetal development, has been identified (17, 23, 40). These proliferating cells do not express skeletal muscle markers, and are the source of Pax-positive satellite cells. During peri-natal development, this population of cells takes up the satellite cell position and starts transcribing Myf5. Therefore, it appears that the Pax3/Pax7-positive cell population gives rise to a committed myogenic precursor. Interestingly, P19[Sox15] cells also express Pax3/7 and Myf5, but not MHC, a marker of differentiated skeletal muscle. It is tempting to speculate that Sox15 is responsible for establishing this population of myogenic progenitor cells.

Although Pax3/7, Meox1 and Foxc1 were expressed in P19[Sox15] cells, they fail to progress through the myogenic pathway. Pax3 has previously been shown to be sufficient to drive MRF and MHC expression under non muscle-inducing conditions (42). However, in P19[Sox15] cells, the upregulation of Pax3 does not lead to terminal differentiation or induce the formation of bipolar myocytes. Using microarray, we attempted to identify genes expressed in P19[Sox15] cell lines which may contribute to the lack of myogenic progression observed. Our screen revealed a dramatic increase in Wnt3a transcripts in P19[Sox15] cells, compared to P19[Control] cells. Furthermore, unlike the expression of Wnt3a during DMSO-induced differentiation, Wnt3a transcripts were not downregulated in the presence of Sox15. Recent studies have demonstrated that the expression of a constitutively active ß-catenin in satellite cells caused an increase in the proportion of cells expressing Pax7, without a concomitant increase in MyoD. These results suggest that ß-catenin promotes self-renewal of muscle precursors, with fewer cells undergoing myogenesis (35). Canonical Wnt signaling, via Wnt3a, also regulates proliferation of satellite cells during regeneration (32). It

is tempting to speculate that the elevated levels of Wnt3a observed in our system are contributing to the maintenance of muscle precursors at the expense of differentiation.

Concomitant with the increase in the expression of genes that regulate cellular proliferation, we observed increases in the myogenic inhibitors Msx1 and Id1. Msx1 is known to block the differentiation of myoblast cell lines *in vitro* (45, 51) and in the developing limbs of mice (2, 20). Msx1 exerts its inhibitory effects by binding Pax3, preventing DNA binding and antagonizing the myogenic activity of Pax3 in migrating limb precursors (2). Ectopic expression of Id1 in NIH 3T3 cells accelerates cell growth and inhibits the expression of p21, a cyclin-dependent kinase inhibitor which prevents cell cycle progression (38). In addition, Id1 has been shown to act as a dominant-negative inhibitor of myogenesis by preferentially binding E-proteins, thereby inhibiting the binding of MyoD/E-protein complexes to E-box containing genes (3, 46). Using bioinformatical approaches, several conserved Sox binding sites were identified within a 25kb region of genomic DNA upstream of Wnt3a, Id1 and Msx1. However, attempts to identify direct binding of Sox15 to these genes were unsuccessful, implying a possible indirect regulation of gene expression by Sox15. Knockdown of Sox15 expression in C2C12 myoblasts also perturbed cell cycle kinetics and caused a decrease in cellular proliferation, further supporting the role of Sox15 in maintaining the proliferating myogenic precursor population (29). Therefore, given the experimental evidence summarized here, the phenotype of P19[Sox15] cells is consistent with a model in which expression of Sox15 is positive towards specification of pre-skeletal mesoderm.

In Chapter 2, we analyzed the phenotype of cells that in which Sox15 function and expression had been altered. The first approach involved the generation of stable cell lines expressing a dominant-negative version of Sox15. Under muscle-inducing conditions, the

presence of the dominant-negative transcription factor resulted in a decrease in muscle precursor markers as well as a decrease in MRF and MHC expression. To extend the results obtained from the dominant-negative studies, knockdown of endogenous Sox15 expression was achieved using shRNA technology. In this case, only Pax3 and Meox1 expression were significantly altered, whereas MRF expression and overall differentiation were unaffected. The more severe phenotype of the dominant-negative mutant is likely a consequence of chromatin remodeling. The chromatin of genes bound by the dominant-negative Sox15 mutant will have undergone remodeling to a conformation that is prohibitive to transcriptional regulation. Therefore, other transcription factors that may also bind and regulate the expression of Sox15-target genes are prevented from doing so, whereas in the absence of Sox15 protein (by shRNA knockdown), genes that may compensate for the lack of Sox15 are free to bind and regulate expression of Sox15 target promoters. It is possible that other genes, including other Sox family members, can compensate for the lack of Sox15 in P19 cells.

Our finding that Sox15 knock-down results in the loss of Pax3 and Meox1 expression, but not overall myogenesis, is consistent with the phenotype of mice lacking Sox15. Examination of skeletal muscle formation in $Sox15^{-/-}$ mutant mice indicates that the process appears to proceed normally during embryonic development (26). However, these animals display a delay in muscle regeneration following crush injury to the TA muscle, shown by a loss of MyoD but not Myf-5 expression, suggesting impairment in satellite cell formation/function (26, 29). There have been conflicting reports regarding the presence of satellite cells in $Sox15^{-/-}$ mice, with one group observing a normal number of satellite cells in mutant myofibers and another group reporting an 80% decrease in satellite cell number in mutant mice, compared to control animals (26, 29). The original knockout of Sox15 was

performed in a 129/C57BL/6 strain of mice and did not lead to any changes in satellite cell number (26). Inactivation of Sox15 in a pure C57BL/6 background dramatically reduced the number of satellite cells detected in the tibialis anterior muscle of mice (29). These observations suggest that an unknown modifier gene expressed in the 129 strain of mice is able to rescue the satellite cell defect in the absence of Sox15. It will be of interest to other researchers to determine the identity of the modifier gene(s). Since Pax3 is an important determinant of satellite cell formation and MyoD regulation, our results in P19 cells are consistent with the $Sox15^{-/-}$ mouse phenotype (7, 39). Interestingly, embryonic expression of Meox1 or Pax3 was not examined in $Sox15^{-/-}$ mice (26). We would predict the downregulation of Pax3 and Meox1 expression in the developing myotome.

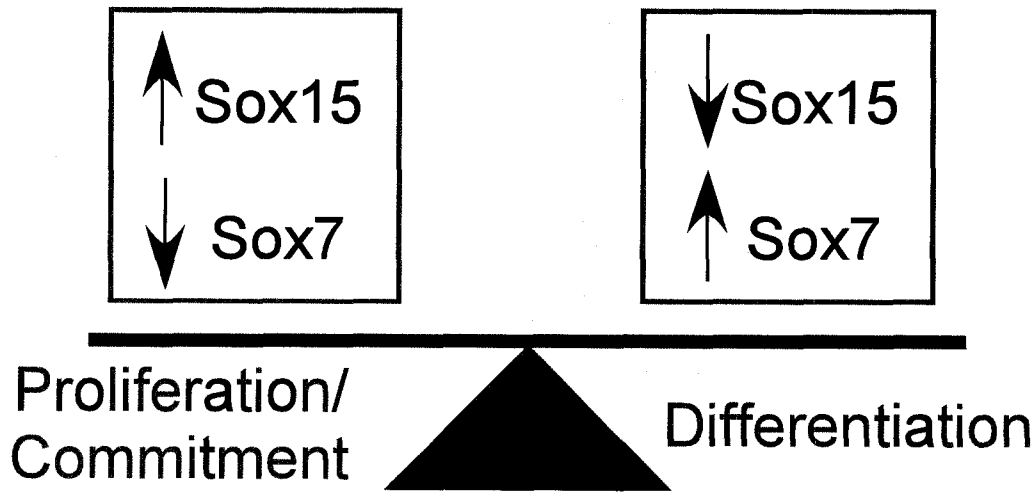
In Chapter 2, a novel role for Sox7 in regulating skeletal muscle differentiation was discovered. Unlike P19[Sox15] cell lines, P19[Sox7] cells are able to enhance the expression of muscle precursor genes, MRFs and MHC. To date, Sox7 has been implicated in the development of endodermal (14, 31, 44) and cardiogenic tissues (8, 18, 34), but has not been reported to be involved in the regulation of skeletal myogenesis. Members of our lab are currently performing knockdown studies in order to confirm the endogenous role of Sox7, since it is possible that Sox7 may be mimicking the activity of another Sox family member. These results will help to clarify the function of Sox7 during myogenesis in P19 cells. Given that both Sox7 and Sox15 are able to control the expression of Pax3/7, Meox1 and Foxc1, but not terminal myogenesis, it would be of interest to identify genes which are differentially expressed between the two cell lines and could account for these differences in activity between the two Sox proteins during the later stages of myogenesis. Global analysis of gene expression could be carried out in order to identify such genes.

Sox15 has also been shown to bind to DNA upstream of Sox7 in P19[Sox15] cells. Interestingly, these cells do not express any detectable levels of Sox7 mRNA, suggesting that Sox15 may negatively regulate Sox7 expression. In such a model, Sox15, bound to the Sox7 promoter, would prevent its expression, allowing the committed muscle precursors to proliferate. During endogenous P19 cell differentiation, Sox15 mRNA levels decrease as myogenesis proceeds. Therefore, it is possible that the decrease in Sox15 expression relieves the inhibition of Sox7, allowing differentiation to take place. It will be of great interest to determine if these observations hold true in mES and hES cell systems. Based on our results, we propose a model where the relative levels of Sox15 and Sox7 control P19 cell proliferation/commitment and differentiation, respectively (Figure 5.1).

5.2 – Control of Muscle Precursor Cell Fate By Signalling Molecules

In Chapters 3 and 4 we report the investigation of the role of secreted signaling molecules in regulating the expression of muscle precursor genes, specifically Sox7 and Foxc1. Canonical Wnt signaling has been shown to be essential for Pax3/7 and Gli2 expression in the developing somite, as well as in P19 cells (6, 21, 33, 37). Here, we expand on that theme and investigate whether Wnt signaling can regulate expression of Foxc1/2 and Sox7, transcription factors expressed in the somites (19, 22, 30, 47, 50). Activation of the Wnt signaling pathway by LiCl inhibition of GSK3β resulted in the expression of Foxc1/2 and Sox7. Although LiCl has long been used as an activator of the canonical Wnt signaling pathway (24), results obtained from such studies must be carefully interpreted since GSK3 has been implicated in regulating several other biological processes including nuclear factor of activated T-cell signaling, insulin signaling and metabolic regulation as well as the regulation of amyloid-beta protein levels (9, 10, 12). Using a dominant-negative β-catenin

Figure 5.1 – Model for the role of Sox15 and Sox7 during skeletal myogenesis. The relative levels of Sox15 and Sox7 are related to the proliferation *versus* differentiation status of P19 EC cells.



mutant to block Wnt signal transduction, we observed a complete absence of Foxc1/2 transcripts and a dramatic decrease in Sox7 expression. Mice null for both Wnt1 and Wnt3a show a loss of the medial compartment of the dermomyotome, as well as a reduction in Pax3 and Myf5 expression (15, 21). Although the expression of Foxc1/2 and Sox7 has not been investigated in these animals, we would predict a decrease/loss in expression, which may possibly contribute to the decrease in Pax3 expression observed. In Chapters 3 and 4, we propose a direct regulation of Foxc1/2 and Sox7 by β-catenin, as demonstrated by the ChIP studies. Based on these observations, it is possible to envision a model where activation of the Wnt signaling pathway results in the concomitant expression of several genes involved in mesodermal patterning and regulation of muscle precursor cell fate. Our studies focused mainly on investigating the effects of β -catenin dependent signaling on the regulation of Foxc1/2 and Sox7 expression, although we cannot dismiss that non-canonical pathways of Wnt signal transduction may also be involved in regulating the expression of these genes.

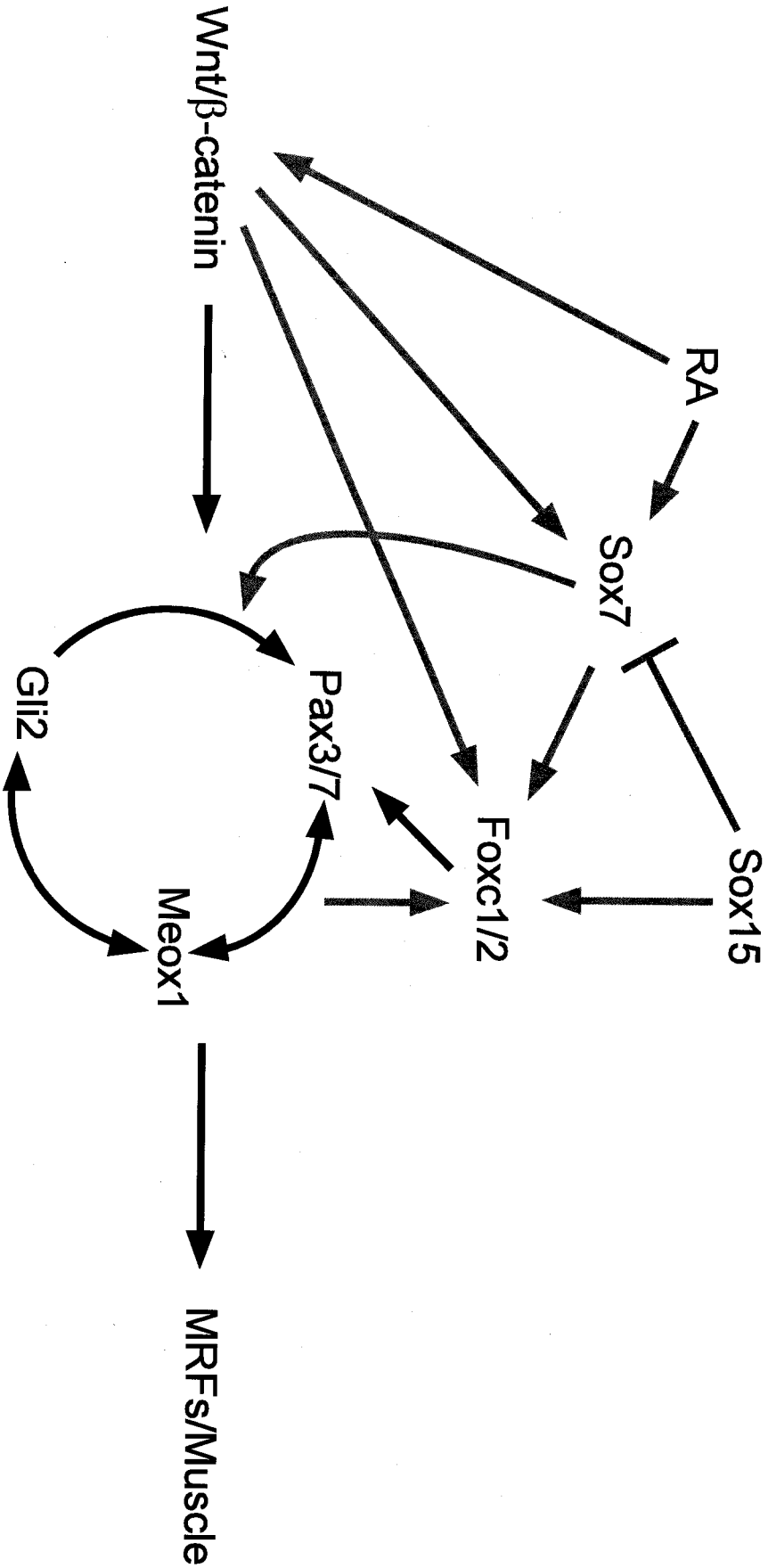
Our lab has previously demonstrated that RA both accelerates and increases the expression levels of genes regulating the specification and commitment of myogenesis, such as Pax3, Meox1, MyoD, and myogenin (Kennedy and Skerjanc, unpublished results). Therefore, RA functions by enhancing the specification of mesoderm cells to the skeletal muscle lineage. The exact mechanisms by which RA induces expression are still unclear, as no direct binding of RAR to the promoters of these genes has been reported. Microarray analysis of these RA-treated cells revealed that Sox7 expression is enhanced following RA exposure (Kennedy, Savage, and Skerjanc, unpublished results). In Chapter 4, we further characterize the expression of Sox7 in RA-treated cells, specifically that Sox7 expression peaks earlier than Pax3 and Meox1, suggesting that the RA-mediated enhancement of those genes occurs via the induction of Sox7. Although hundreds of genes have been reported to

be RA-inducible, only a small percentage of these have been shown to contain functional RAREs. *In silico* analysis of the Sox7 genomic DNA sequence has identified one conserved RARE approximately 25kb upstream of the transcriptional start site. Studies, performed by other students in the lab, are currently underway to determine if RAR directly binds the Sox7 gene. During development, signal transduction pathways do not function as isolated linear conduits for the transmission of information, but rather function as networks, integrating with each other at multiple levels (reviewed in 49). The RA signaling pathway has been shown to interact with the Wnt pathway, negatively regulating its activity. The RA-RAR complex has been demonstrated to compete with TCF4 for binding to β-catenin, thereby sequestering the available pool of nuclear β -catenin and blocking its ability to activate Wnt target genes in MCF-7 breast cancer cell lines (13). In addition, exposure to exogenous sources of RA antagonizes the dorsalizing effects of LiCl in Xenopus embryos (27), suggesting that a balance of Wnt and RA signaling is required for proper embryonic development. Given that the Wnt and RA pathways have been known to intersect under certain circumstances, we examined, in Chapter 4, whether RA functions upstream or downstream of the Wnt signaling pathway. We have demonstrated in Chapter 4 that RA appears to act upstream of Wnt3a and cannot enhance Sox7 expression in the presence of a dominant negative β-catenin. However, we cannot disregard the possibility that the RA-bound RAR/RXR heterodimer may bind to the regulatory regions of the Sox7 gene during myogenesis, but are prevented from positively regulating transcription in the presence of the dominant-negative β-catenin. Therefore, based on our observations, we cannot positively conclude that RA only acts upstream of Wnt signaling.

5.3 – Summary and Future Perspectives

In this thesis, we have provided further insight into the mechanisms that regulate the specification of future myogenic cells. We have discovered a novel role for two members of the Sox protein family, Sox15 and Sox7, in controlling the early step of myogenic commitment. In addition, Sox7 is able to positively regulate terminal differentiation of myoblasts into myocytes. We propose a model where the levels of Sox15 and Sox7 expression could act as a kind of thermostat controlling the cell's decision to proliferate or differentiate. Further studies aimed at identifying genes which are differentially expressed between P19[Sox15] and P19[Sox7] cells will help us better understand how myogenic commitment and differentiation are controlled at the molecular level. Furthermore, genome-wide transcription factor binding profiling will help us identify genes that are under the direct control of Sox15 and Sox7. Finally, we also examined the effect of known signaling pathways (Shh, Wnt, RA) on the expression of genes expressed in the muscle precursor cells. The data presented in Chapters 2, 3 and 4 allow us to build a more comprehensive map of the molecular events that regulate myogenesis in P19 cells (Figure 5.2). This information can potentially be used to devise new therapeutic interventions aimed at repairing dystrophic muscle.

Figure 5.2 – The molecular hierarchy of genes that regulate myogenic commitment in P19 cells. A schematic depiction of the genes expressed during skeletal muscle differentiation in P19 cells. The green arrows in the network represent the positive interactions we have uncovered, whereas negative regulation of gene expression is shown by the red arrows. The black arrows represent findings from previously published studies (35, 36, 41, 42).



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Appendices

α-actin	F: CTGGTATTGCCGATCGTATG R: CTTGCTGATCCACATTTGCT	Sox7	F: ACCTCAGGGGACAAGAGTTCG R: GTTTTTCTCQGGCQGCGTGTTC
Foxc1	F: CAAGACGGAGAACGGTACGTG R: GGCTCTCGATTTTGGGCACT	Sox15	F: CAACTATTCGACAGCCTACCTGCC R: GTGTTTAGTGTGCATTCTGGTTCC
Foxc2	F: AACCCAACAGCAAACTTTCCC R: GCGTAGCTCGATAGGGCAG	Wnt3a	F: TGGCTGAGGGTGTCAAAGC R: CGTGTCACTGCGAAAGCTACT
GAPDH	F: TCGGTGTGAACGGATTTG R: GGTCTCGCTCCTGGAAGA	Wnt5a	F: TGCGGAGACAACATCGACTAT R: TCCATGACACTTACAGGCTACA
Gli2	F: CAACGCCTACTCTCCCAGAC R: GAGCCTTGATGTACTGTACCAC	Pax3 promoter	F: ACATGGAGAGAGGGTTGGAGGATT R: AACGTCTTGGCTAGGAGCTGAAGT
Id1	F: CCTAGCTGTTCGCTGAAGGC R: CTCCGACAGACCAAGTACCAC	Sox7 promoter	F: ACAGTCTGCATCACTGCTCA R: ACCAACTAGAGGGGCAGGAA
Meox1	F: TGGCCTATGCAGAATCCATTCC R: TGGATCTGAGCTGCGCATGTG	Fox-ChIP-A	F: TGGCTCATTCACAGGCGTCTCT R: GCTAAGGCGGCCAAATAACCAG
MHC	F: ACAACCCCTACGATTATGCGT R: ACGTCAAAGGCACTATCCGTG	Fox-ChIP-C	F: GGCAGTTTGGCTCTTCATCTACCT R: TGGTTGAATTAATCAGATAGTCGTCCA
Msx1	F: TGCTGCTATGACTTCTTTGCC R: GCTTCCTGTGATCGGCCAT	Fox-ChIP-D	F: TAAAGCCGTAGGTGTGCATGGTCT R: TTTCTCGGGCGGCAACTTTCACTT
Myf5	F: CCTGTCTGGTCCCGAAAGAAC R: GACGTGATCCGATCCACAATG	Fox-ChIP-F	F: CTCACGTGCAAGATGCAGAT R: AATCCGTTTGGTAGGCTGTG
MyoD	F: CCCCGGCGGCAGAATGGCTACG R: CGCTGGTCGCTGGAGAG	GAPDH-ChIP	F: AGTGCCAGCCTCGTCCCGTAGACAAAATG R: AAGTGGGCCCCGGCCTTCTCCAT
Myogenin	F: GCAATGCACTGGAGTTCG R: ACGATGGACGTAAGGGAGTG	LBE1	F: GAGTGGAGCAAGCCAATAGC R: TTGTTGCTGTTTGTCCCTTG
Pax3	F: TTTCACCTCAGGTAATGGGACT R: GAACGTCCAAGGCTTACTTTGT	LBE2	F: CCTTGCACGTTGTAAGCAAA R: CAGGCGGTGATGAGGTAAAT
Pax7	F: CTCAGTGAGTTCGATTAGCCG R: AGACGGTTCCCTTTGTCGC		

Appendix A: Primer sequences used for SYBR Green Q-PCR Analysis

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2008 (Sept-Dec) **Replacement Lecturer**
Department of Biochemistry, Microbiology and Immunology
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POST SECONDARY EDUCATION AND DEGREES

2002 – 2008 **PhD Biochemistry**
University of Ottawa
Ottawa, Ontario, Canada

1998 – 2002 **BSc Honours Cell Biology**
University of Western Ontario
London, Ontario, Canada

SCHOLARSHIPS AND AWARDS

2008 First place, Graduate Symposium Day, University of Ottawa

2006 Travel Award, Canadian Student Health Research Forum, Winnipeg, MB

2006 Travel Award, Department of Biochemistry, University of Ottawa

2006 3rd prize, PhD Presentation, Biochemistry Graduate Research Symposium, University of Ottawa

2005-2008	Excellence award – University of Ottawa
2005-2007	CIHR Institute of Aging Doctoral Research Award
2005-2007	OGSST Scholarship (declined)
2005	Travel Award, Department of Biochemistry, University of Ottawa
2004	ASBMB Travel Award, Il Ciocco, Italy
2004	Nomination, Teaching Assistant of the Year, University of Western Ontario
2003-2005	Excellence Scholarship, University of Western Ontario
2003-2005	NSERC – Postgraduate Scholarship A
2003-2004	OGS Scholarship (declined)
2003	Chair's Travel Award, The University of Western Ontario
2003	Merck Frosst Travel Award (CSBMCB), Montreal, QC

RELATED WORK AND TEACHING EXPERIENCE

2006	Revising and editing of undergraduate Molecular Biology lab manual (French & English), Faculty of Science, University of Ottawa
2006	Teaching Assistant (BCH 4125 – Cellular Regulation and Control), Faculty of Science, University of Ottawa
2005	Pre-lab lecturer (BCH 3356 – Molecular Biology Laboratory and BCH 3756 Laboratoire de biologie moléculaire), Faculty of Science, University of Ottawa
2003-2004	Teaching Assistant (BIO 022 – Introductory Biology Lab), Faculty of Science, University of Western Ontario, London, ON

SCHOLARLY AND PROFESSIONAL ACTIVITIES

Contributions to Scientific Training

2005-2006	Mentor for undergraduate student, University of Ottawa
2003-2004	Mentor for undergraduate student, University of Western Ontario

Scientific Outreach and Advocacy

2005	Judge, Intermediate/Senior Biotechnology Division, Ottawa District Science Fair, Ottawa, ON
2003-2004	Graduate Student Representative, Basic Science Gender Issues Committee Faculty of Medicine, University of Western Ontario
2002-2004	Volunteer, Let's Talk Science Partnership Program University of Western Ontario

PUBLICATIONS

Refeered Papers, in press, submitted or in preparation

1. **Savage, J.,** Conley A.J., Blais A. and Skerjanc, I.S. Sox15 and Sox7 differentially regulate the myogenic program in P19 cells (*Manuscript in revision with Stem Cells*)
2. **Savage, J.,** Voronava, A. and Skerjanc, I.S. Canonical Wnt signaling regulates Foxc1/2 expression in P19 cells. (*Manuscript submitted to Differentiation*)
3. Gianakopoulos, P.J., **Savage, J.**, Waddington, M.L., and Skerjanc, I.S., The myogenic regulatory factors regulate transcription factors of the somite. (*Manuscript in preparation*)
4. Kennedy, K.A.M., Porter, T. , Mehta, V., Price, F. , Ryan, S., Karamboulas, C., **Savage, J.**, Drysdale, T., Bennett, S. and Skerjanc, I.S., Bone Morphogenetic Protein 4 and Retinoic Acid function antagonistically in regulating cardiac and skeletal muscle development. (*Manuscript in preparationn*)
5. McKinney, J.L, Murdoch D.J., Wang J, Robinson, J., Biltcliffe, C., Walker, P.M., **Savage, J.**, Skerjanc, I, and Hegele, R.A. Venn analysis as part of a bioinformatic approach to prioritize expressed sequence tags from cardiac libraries. *Clinical Biochemistry 2004, 37(11):953-60*

Conference Abstracts

1. Kennedy, K. A. M., Porter, T. , Mehta, V., Ryan, S., Price, F., Karamboulas, C., **Savage, J.**, Bennett, S.A., and Ilona S. Skerjanc. Retinoic acid can enhance skeletal muscle specification and bypass inhibition by Bone Morphogenetic Protein 4 but not by a dominant negative ß-catenin, Keystone Symposia: Tumor Suppressors and Stem Cell Biology, Vancouver, Canada, February 2008.

2. Kennedy, K. A. M., Porter, T. , Mehta, V., Price, F., Karamboulas, C., Savage, J., Rudnicki, M., and Skerjanc , I.S. Retinoic acid can enhance skeletal muscle

specification and bypass inhibition by Bone Morphogenetic Protein 4 but not by a dominant negative ß-catenin, Myogenesis, Gordon Research Conference, Il Ciocco, Italy, May 2007.

3. **Savage, J** and Skerjanc, I.S. SOX transcription factors regulate pre-skeletal mesoderm formation. CIHR National Student Research Poster Competition, June 7th, 2006 University of Winnipeg, Winnipeg, Manitoba.

4. **Savage, J.**, Conley, A.J, Boisvenue, S. and Skerjanc, I.S. Regulation of Pax3 expression by the SOX family of transcription factors. Frontiers in Myogenesis, April 27 - May 2, 2006, Pine Mountain, Georgia, USA

5. Gianakopoulos, P., Sleiman, M., **Savage, J.**, and Skerjanc, I.S., The myogenic regulatory factors regulate transcription factors of the somite. Frontiers in Myogenesis, Mountain Creek Conference Center, Callaway Gardens, Pine Mountain, Georgia, USA, April 27-May 2, 2006.

6. **Savage, J.** and Skerjanc, I.S. Examining the role of FOXC1 during muscle differentiation in P19 cells. EMBO/FEBS Workshop: The Molecular and Cellular Mechanisms underlying skeletal muscle formation and repair, September 24-29 2005, Fontevraud, France.

7. **Savage, J.**, Conley, A.J. and Skerjanc, I.S. Sox15 regulates Pax3 expression during skeletal myogenesis in P19 cells. 2004 Gordon Research Conference on Myogenesis, May 16-21 2004, Il Ciocco, Italy.

8. Conley, A.J., **Savage, J.** and Skerjanc, I.S. Sox15 Induces Pax3 Expression in P19 Embryonal Carcinoma Cells. 24th Annual Great Lakes Mammalian Development Meeting, March 6-7, 2004, Mount Sinai Hospital, Toronto, Canada

9. **Savage, J.** and Skerjanc, I.S. Regulation of Pax3 Expression During Myogenesis in P19 Cells. 24th Annual Great Lakes Mammalian Development Meeting, March 6-7, 2004, Mount Sinai Hospital, Toronto, Canada

10. **Savage, J.** and Skerjanc, I.S. Regulation of Foxc1 Expression by Pax3, Gli2 and β-Catenin During Myogenesis. HUPO 2nd Annual & IUBMB XIX Joint World Congress, Montreal, October 8 -11, 2003